TARGET ANTIGENS IN CANINE IMMUNE-MEDIATED HEMOLYTIC ANEMIA

A Thesis

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Primary immune-mediated hemolytic anemia (IMHA) is an important cause of serious morbidity and mortality in dogs. Despite numerous studies examining the demographics, treatment options, and prognostic indicators of disease, the mechanisms that underlie immune dysregulation remain poorly understood. The purpose of this study was to directly identify unique erythrocyte membrane antigens in dogs diagnosed with primary IMHA. Blood samples were obtained from dogs presented to the Ontario Veterinary College Teaching Hospital with primary IMHA prior to treatment, and also from control dogs (healthy dogs and dogs with non-immunologic anemia). Antibodies bound to erythrocyte membranes were eluted using xylene. Immunoblots using patient eluates reacted against pooled canine erythrocyte lysates, and autologous patient plasma reacted against xylene eluates, were performed. These results were compared to results of similar experiments using samples from control dogs. Bands appearing in patient but not control samples were considered potential autoantigens, and were submitted for identification by liquid chromatography followed by tandem mass spectrometry. Samples from 13 dogs with primary IMHA, 4 dogs with non-immunologic anemia, and 2 healthy dogs, were analyzed. Immunoblotting confirmed the presence of immunoglobulin in
eluates from all dogs. Semi-quantitatively, eluates from IMHA patients contained more immunoglobulin than those of control dogs. Mass spectrometry identified complement C3 in patient but not in control dog samples. Additional peptides identified by mass spectrometry in patient but not control dog samples included peroxiredoxin 2 and calpain. The former comprises a cytosolic hydrogen peroxide scavenger, and has been associated with erythrocyte membranes under oxidative stress conditions inducing spherocytosis. Calpain is a calcium-dependent protease that may become activated with oxidative stress and induce erythrocyte apoptosis. These findings suggest that oxidative stress and apoptosis contribute to the pathogenesis of canine IMHA.
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<tr>
<td>aPTT</td>
<td>activated partial thromboplastin time</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte antigen 4</td>
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<td>DEA</td>
<td>dog erythrocyte antigen</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>FDP</td>
<td>fibrin degradation product</td>
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<td>HCT</td>
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<td>HRP</td>
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<td>Ig</td>
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<td>IMHA</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>nTreg</td>
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<tr>
<td>PBS</td>
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<td>PMSF</td>
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<td>SDS PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>TCR</td>
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LITERATURE REVIEW

Canine Primary Immune-mediated Hemolytic Anemia

Immune-mediated hemolytic anemia (IMHA) is one of the most important causes of severe anemia and serious morbidity in dogs in North America (Feldman, 1996; Scott-Moncrieff et al, 2001). Neoplasia, infection, and even drug therapy can trigger destruction of red blood cells (RBCs) by the patient’s own immune system. However, in the majority of cases, an underlying cause cannot be found. Such cases are referred to as primary or idiopathic IMHA (Carr et al, 2002; Scott-Moncrieff et al, 2001). In primary IMHA, disease is thought to arise as a consequence of breakdown of tolerance and elicitation of specific, adaptive immune response against self-antigens (Fagiolo et al, 2003; Semple & Freedman, 2005). The result is the production of inappropriate antibodies that target antigens in the patient’s own RBC membrane. Anemia ensues because RBC lifespan is shortened – either by accelerated erythrophagocytosis of antibody-coated RBCs, and/or by complement-mediated lysis of the RBC itself (Thrall et al, 2004; Weinkle et al, 2005).

Clinical and Laboratory Features

Primary IMHA typically affects middle-aged dogs, and includes the following classical clinical and laboratory signs: acute onset lethargy, exercise intolerance, pallor and/or jaundice, occasional abdominal pain or vomiting, mild to severe regenerative anemia, autoagglutination, spherocytosis, occasional ruptured erythrocyte membranes (ghosts), hyperbilirubinemia, and inflammatory leukogram (including neutrophilia with left shift and monocytosis) (Nelson & Couto, 1998; Stockham & Scott, 2008). A positive Coombs’ test supports the diagnosis of IMHA; however, since the direct Coombs’ test
may be negative in up to 30-40% of dogs with disease (Quigley et al, 2001; Warman et al, 2008), it is not used as a sole criterion for diagnosis (Nelson and Couto, 1998; Wardrop, 2005). In severe cases, anemia progresses rapidly, and dogs may present in shock (Al-Ghazlat, 2009). Current therapy consists of supportive care and non-specific suppression of the immune response (Grundy & Barton, 2001; Mason et al, 2003; Scott-Montcrieff et al, 2001; Weinkle et al, 2005; Piek et al, 2008). Treatment can therefore be thought of as being largely palliative (i.e., controlling signs of disease), rather than curative (i.e., addressing the mechanisms underlying immune dysregulation). Moreover, current immunosuppressive strategies are associated with certain risks: for example, immunosuppressive doses of glucocorticoids - the mainstay of IMHA treatment - predispose the patient to a variety of serious and potentially fatal complications, including severe infection and thromboembolic disease (Carr et al, 2002; Scott-Montcrieff et al, 2001; Semple & Freedman, 2005; Weinkle et al, 2005). Regardless of treatment, at present, canine primary IMHA carries a high mortality rate, ranging from 23% to 70%, with death usually occurring within the first 2 to 3 weeks of treatment (Grundy & Barton, 2001; Mason et al, 2003; Scott-Montcrieff et al, 2001; Weinkle et al, 2005; Piek et al, 2008). Clearly, more effective therapies - which specifically target dysfunctional immunoregulatory mechanisms without causing nonspecific immune suppression and unwanted adverse effects - are an unmet, and urgent, need.

Pathogenesis

Although the clinical and laboratory features of primary IMHA are well-characterized, the exact initiating events that lead to failure of immunoregulation are poorly understood (Al-Ghazlat, 2009). Autoimmunity is thought to arise via one of
several mechanisms which, in the context of spontaneous autoimmune disease, are probably not mutually exclusive: (1) loss of “immunologic ignorance;” (2) loss of peripheral tolerance; and (3) loss of central tolerance.

Immunologic ignorance is a state where antigen and lymphocyte bearing cognate T-cell receptors (TCRs) never interact in such a way as to induce an immune response (Salaman, 2003). Certain tissues contain immuno-privileged sites that sequester potentially auto-reactive antigen (e.g. CNS, anterior chamber of the eye, testis). However, proteolytic tissue destruction from chronic inflammation may serve to expose these antigens, either by providing an excess of antigen for presentation and/or by unmasking cryptic epitopes (Bei et al., 2008). Additionally, infection may bring about loss of ignorance by molecular mimicry, by massive polyclonal B or T cell activation, or by inducing co-stimulatory activity as a consequence of inflammation (Salaman, 2003; Fagiolo and Toriani-Terenzi, 2003). To date, conclusive evidence linking chronic infections or inflammatory processes to development of primary IMHA is lacking.

**Regulatory T cells**

There is growing evidence for the role of various subsets of regulatory T cells in actively suppressing self-reactive T cells that have escaped central tolerance (discussed later), and thus prevent the onset of autoimmunity (Andre et al., 2009; Piccirillo et al., 2008). Amongst the best-studied thus far are the “natural” CD4+CD25+ T regulatory cells (nTreg). These cells acquire their immunoregulatory functions intra-thymically, and constitute roughly 1-10% of total CD4+ T cells within lymphoid compartments (Piccirillo et al., 2008). They constitutively express the forkhead box P3 (Foxp3) gene, which encodes a forkhead winged helix transcriptional factor (FoxP3) essential for
immunoregulatory function (Zhou et al., 2009). CD4+CD25+FoxP3+ nTreg home to lymphoid organs, and suppress *in vivo* and *in vitro* responses of naïve T cells, dendritic cells, macrophages, NK cells and B cells by a variety of mechanisms (Andre, 2009; Piccirillo, 2008). Abundant evidence indicates that functional nTreg are important in preventing autoimmunity. Firstly, Sakaguchi and colleagues (1995) demonstrated that mice depleted of nTreg developed antibodies against double-stranded DNA and developed SLE-like disease, and perhaps more importantly, disease was reversed by adding CD4+CD25+ nTreg. Secondly, mice infused with *ex vivo* expanded nTregs (adoptive transfer) during induction of autoimmunity show a decrease in the severity of disease, including one model of murine AIHA (Mqadmi et al., 2005; Andre et al., 2009). Thirdly, congenital deficiency in nTreg in mice and humans due to *foxp3* loss-of-function mutations results in a condition known as IPEX syndrome (polyendocrinopathy, enteropathy, X-linked), which is characterized by severe, early-onset multi-organ autoimmunity (Riley et al., 2009). And finally, nTregs isolated from patients with spontaneously-occurring autoimmune diseases (e.g. multiple sclerosis, SLE, type 1 diabetes, psoriasis, myasthenia gravis, immune-mediated thrombocytopenia, and rheumatoid arthritis) appear to show functional defects such as reduced FoxP3 expression, impaired survival, inability to suppress conventional T cells, or reduced expression of the inhibitor molecule CTLA-4 (Andre et al., 2009, Riley et al., 2009, Piccirillo et al., 2008). Unfortunately, at this time nTreg in dogs are not as well-characterized as they are in mice and humans, and similar studies exploring the role(s) of nTreg in canine primary IMHA are lacking.
MHC

The major histocompatibility complex (MHC) is one of the most extensively studied regions in the human genome, and strong associations of particular MHC haplotypes with almost every autoimmune disease, including type I diabetes, systemic lupus erythematosus, inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis, have been demonstrated (Fernando et al, 2008). A popular notion is that differences in antigen presentation conferred by various MHC haplotypes may result in differences in affinity of binding with presented antigen and with cognate TCRs; in cases of sub-optimal (i.e. low affinity) binding, potentially auto-reactive TCRs may escape thymic negative selection. In short, certain MHC haplotypes may contribute to failure of central tolerance and increase risk of autoimmunity (Fernando et al, 2008). Joosten and colleagues (1994) demonstrated in Lewis rats that immunodominant peptides implicated in various models of autoimmunity (e.g. myelin basic protein in experimental autoimmune encephalomyelitis; acetylcholine receptor (AChR) peptide in experimental autoimmune myasthenia gravis; and interphotoreceptor retinoid binding protein in experimental anterior uveitis) have low-intermediate to poor binding affinity to their MHC II restriction elements, relative to tolerogenic self peptides. Further to this finding, Liu and colleagues (1995) showed that altering the MBP epitope implicated in experimental autoimmune encephalomyelitis (EAE) at position 4 (from lysine to alanine or tyrosine) in H-2u mice improves binding affinity to the MHC II restriction element, I-Au, and results in thymic deletion of CD4+CD8- cells bearing encephalitogenic TCR.

Could such a mechanism, involving poor affinity of immunodominant epitopes of RBC membrane proteins to MHC restriction elements, be implicated in canine primary IMHA? Recently, Kennedy and colleagues (2006) found significant associations between
certain dog leukocyte antigen (DLA) haplotypes and risk of primary, Coombs positive IMHA. Two DLA haplotypes in particular were increased in frequency in patient groups (versus control) with IgG-mediated IMHA: DLA-DRB1*00601/DQA1*005011/DQB1*00701 and DLA-DRB1*015/DQA1*00601/DQB1*00301. However, several issues remain unresolved and hamper our ability to draw similar potential causal relationships between DLA, failure of central tolerance, and canine IMHA. Firstly, due to the high degree of linkage disequilibrium seen amongst certain breed groups (analogous to certain racial groups and isolated human populations), parsing out other potential “causal” genes co-segregating with MHC loci is difficult, if not impossible, thus the associations between MHC haplotypes and disease risk do not prove causality (Sospedra et al., 2006). Finally (and most obviously), little work has been done to discover RBC autoantigens (let alone immunodominant epitopes) targeted in canine primary IMHA, and at present these autoantigens remain largely unknown.

**Previous studies on RBC antigens in dogs with IMHA**

A comprehensive search of the medical and veterinary databases as far back as two decades revealed only two studies by the same group, which attempted to determine target antigens in canine IMHA. Barker and colleagues (1991) immunoprecipitated ether eluates from red cells of seven IMHA dogs with biotin-labeled RBC membrane proteins, and they found at least 2 distinct patterns of immunoreactivity. Ether eluates from two dogs did not show any immunoreactivity; that of one dog showed immunoreactivity against band 3; and those of four dogs showed immunoreactivity against 42 kDa and 29 kDa bands, which the authors suggested were canine glycophorins. In this initial study,
there was no mention of inclusion criteria other than compatible laboratory data and positive direct enzyme-linked antibody test (DELAT), thus the possibility of inclusion of patients with secondary IMHA cannot be ruled out (indeed, one of the patients included was listed as also having concurrent bronchitis). In a follow-up study involving four dogs with IMHA, Barker and Elson (1995) found that ether eluates from 2 dogs were reactive against purified canine glycophorins. Similar to the first study, samples from 2 dogs did not show any immunoreactive bands. The authors surmised that in these 2 cases, the ether elution technique may have failed to recover antibodies from these patients, or perhaps there was a failure in biotin labeling of RBC membrane. In addition, neuraminidase treatment of antigen immunoprecipitated by eluates from these dogs resulted in a shift in apparent molecular mass on sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE) that would indicate sialic acid removal, and the change in molecular mass was identical to the molecular mass change seen after neuraminidase treatment of purified canine glycophorins. These findings corroborated results of the previous study by Barker and colleagues (1991), and the authors concluded that glycophorins are likely a target autoantigen in some but not all cases of canine IMHA. However, both studies were hampered by small sample size, and there was no attempt to assess reactivity with other RBC membrane proteins in the patients that lacked apparent reactivity against glycophorins.

**Additional animal models of IMHA**

Autoimmune hemolytic anemia (AIHA) in the New Zealand Black (NZB) mouse is a well-studied and classic example of spontaneous, antibody-mediated autoimmune disease (Diiulio et al., 1997; Shen et al., 2003; Hall et al., 2007). AIHA in the NZB mouse
shares several features to primary IMHA in the dog: it is not associated with drugs, infection, or other identifiable factors; it develops spontaneously around 5 to 6 months of age (roughly middle-age for a mouse); extravascular hemolysis (erythropagocytosis of RBCs) predominates, and autoantibody response is helper T cell dependent (Hall et al., 2007). The target autoantigen in murine AIHA is well-characterized, and appears to be band 3 protein (anion exchanger 1), based on western blot analysis of RBC eluate reactivity (Barker et al, 1993). As other studies in human and murine models of autoimmunity demonstrate (including those by Liu et al and Joost et al cited above), elucidation of target autoantigens in AIHA is an important step towards understanding mechanisms leading to development of autoimmunity. Hall and colleagues (2007) generated AE1\(^{-}\) NZB mice that did not express band 3 protein on RBCs, and found that development of AIHA was not dependent on expression of band 3. AE1\(^{-}\) NZB mice developed AIHA similarly to their wild type counterparts, produced autoantibodies in roughly the same concentration as wild type NZB, but they generated RBC autoantibodies with other specificities (mostly to the glycophorins). The results of this study support the view that failure of active tolerance mechanisms, which are independent of autoantigen, underlies development of AIHA. The question then arises, why is band 3 the dominant target? The investigators surmised that dominance simply reflected the abundance of a particular antigen: In the case of AIHA, autoaggression starts with the most abundant RBC membrane protein, band 3, and can “switch” to the next most abundant, the glycophorins, when band 3 is unavailable (Hall et al, 2007; Barker et al, 1991).
Tolerance and autoantigens

As the studies in human and murine models of autoimmunity demonstrate (including those cited previously), elucidation of target autoantigens is an important step towards understanding mechanisms which underlie breakdown of self-tolerance, and is an avenue of research critically lacking in canine IMHA. Additionally, knowledge of dominant autoantigens can lead to the development of targeted immunotherapies (Shen et al, 2002). Mucosal tolerance is one such example: It involves the oral or nasal administration of target autoantigen relevant to the disease in question, wherein the autoantigen serves as a “tolerizing” agent (Faria & Weiner, 2006; Larche & Wraith, 2005). Successful therapy using this strategy has been documented in several murine models of autoimmunity, including EAE, diabetes mellitus, arthritis, myasthenia gravis, and autoimmune uveitis (Faria & Weiner, 2006). Based on the promising outcome in animal models and the relative safety, numerous trials in humans have been initiated for the above autoimmune diseases (Barnett et al., 1998; Faria & Weiner, 2006). The mechanisms of oral tolerance are likely complex and have yet to be clarified, but it is believed that induction of antigen-specific regulatory T cells play an important role in the suppression of pathologic immune response, via the production of regulatory cytokines (Larche & Wraith, 2005; Faria & Weiner, 2006).

Several groups have also been investigating the use of altered peptide ligands (APLs) in the treatment of autoimmune disease (Nicholson et al, 1997; Larche & Wraith, 2005; Shen et al, 2003). APLs are similar to the native, autoimmune disease-inducing peptide except for substitutions in one or a few amino acids. These altered peptides, or T cell antagonists, bind MHC receptors similarly to native disease-producing peptides, but deliver antagonist or partial agonist signals, thereby modifying T cell activation and
cystokine production. They are given parenterally, and have been used successfully in the treatment and prevention of murine EAE and AIHA in the NZB mouse (Shen et al, 2003). This strategy, however, requires identification not only of the target autoantigen, but also its immunodominant epitopes (Larch & Wraith, 2005; Shen et al, 2003), which are currently unknown in canine primary IMHA.

**Methods to investigate RBC antigens in immune hemolytic anemia**

**Elution**

Studies investigating RBC autoantigens in immune hemolytic anemias in rats, humans and dogs have all begun with removal of autoantibodies from erythrocytes by one of several elution techniques (Barker et al, 1991; Barker et al, 1993; Barker & Elson, 1995; Iwamoto et al, 2001; Leddy et al, 1994). RBC elution was originally developed to investigate the presence and specificities of alloantibodies on RBCs of human patients who had previously received non-compatible blood transfusions (Chan-Shu & Blair, 1979; Judd, 1993). Elution involves neutralization or disruption of antigen-antibody interactions, which can be comprised of electrostatic forces, hydrogen or ionic bonds, hydrophobic interactions, and van der Waals interactions (Kindt et al, 2007; Judd, 1993). The most commonly employed techniques encountered in the literature include heat (which causes conformational changes to proteins, leading to loss of structural complementarity); sonication (high frequency sound waves which literally shake off antibodies on RBCs); acid elution (extreme changes in pH causing loss of tertiary structure of proteins); addition of chaotropic ions (Cl, I or SCN ions are added, bind to charged amino acid groups, disrupt tertiary structure, and thereby weaken coulombic
attraction between antigen and antibody); freeze-thaw (which relies on the formation of ice crystal-mediated RBC lysis and conformational changes in membrane structures); and use of organic solvents (such as ether, chloroform, or xylene, which interfere with van der Waals interactions between antigen and antibody) (Judd, 1993).

The extracted material or eluate contains immunoglobulin of interest, as well as other non-immunoglobulin proteins (e.g. denatured hemoglobin). No single technique is 100% efficient in extracting RBC-bound antibodies (Judd, 1993). Dumaswala and colleagues (1994) suggested that the total antibody protein concentration comprises less than 1 percent of total eluate proteins obtained by xylene and acid elution techniques in sensitized red cells. In addition, most of the techniques described also result in the destruction of the RBCs (Judd, 1993) or even denaturation of antibody (e.g. heat elution) if care is not taken (Judd, 1993). In a comparison of 11 different techniques, including digitonin-acid, dichloromethane, chloroform, ether, freeze-thaw, xylene, and heat treatment, South and colleagues (1986) showed that xylene elution was the most efficacious in recovering antibodies bound to previously-sensitized RBCs. Furthermore, the authors noted that the xylene technique, as described originally by Chan-Shu and Blair (1979), is relatively rapid and inexpensive, but is hampered by the fact that xylene is potentially carcinogenic and flammable.

Investigation of antibody specificities

Barker and colleagues (1993) examined the specificity of RBC autoantibodies in ether eluates of NZB mice with AIHA using an immunoprecipitation technique followed by western blot. Washed RBCs from Coombs’-negative NZB mice were biotinylated and sensitized with antibody from RBC eluates of mice with AIHA. The sensitized RBCs
were then lysed, and solubilized immune complexes were immobilized on Sepharose-protein A beads, eluted into gel sample buffer, and analyzed by SDS-PAGE followed by western blot (avidin conjugated with peroxidase was used as detection system). RBC eluates from AIHA mice immunoprecipitated a 105 kDa band, which was subsequently identified as band 3 by additional western blot experiments probing specifically with anti-mouse band 3 antibodies. A similar technique (ether elution followed by immunoprecipitation and western blot analysis) was used to study specificity of autoantibodies from dogs with IMHA (Barker et al, 1991; Barker and Elson, 1995), except that RBC lysates (erythrocyte ghosts) were immunoprecipitated with eluate instead of whole red cells.

In the murine model of AIHA, further characterization of the immunodominant epitope has been performed (Shen et al, 2002). Affected NZB mice, but not H2-matched healthy mice, have splenic T cells specific for band 3. These T cells proliferated *in vitro* when exposed to a panel of synthesized 15- to 20-mer peptides spanning the sequence of band 3; however, band 3 peptide 861-874 consistently elicited the most vigorous responses (Perry et al, 1996).

**Hypothesis**

Based on studies of immune-mediated diseases in other species (e.g. autoimmune hemolytic anemia in mice, multiple sclerosis in humans, and type 1 diabetes in humans), we suspect that a common target antigen or a few common target antigens may be involved in canine primary IMHA. Therefore, we hypothesize that the immune response in canine primary IMHA is directed at shared RBC membrane antigens. Further, we hypothesize that these antigens include membrane integral proteins such as glycophorins.
and/or anion exchange proteins. By uncovering the identity of this or these antigen(s), we hope to further our understanding of how primary IMHA arises. The results of the current study would provide a foundation for future work that might result in the characterization of specific dominant epitopes involved in canine IMHA, and potentially to the development of specific, targeted immunotherapies.

**Specific objectives of project**

The goals of this study were (1) to characterize RBC membrane proteins from a pool of healthy dogs using one-dimensional SDS-PAGE; and (2) to directly identify erythrocyte membrane antigens that are targeted by the immune system in dogs diagnosed with primary IMHA.
RESEARCH STUDY

Introduction

Immune-mediated hemolytic anemia (IMHA) is one of the most important causes of severe anemia and serious morbidity in dogs in North America (Feldman, 1996; Scott-Moncrieff et al, 2001). A variety of underlying diseases such as neoplasia and infection, as well as certain drugs can trigger destruction of red blood cells (RBCs) by the patient’s immune system. However, in the majority of cases, an underlying cause cannot be found; such cases are referred to as primary or idiopathic IMHA (Carr et al, 2002; Scott-Moncrieff et al, 2001). Although the clinical and laboratory features of primary IMHA are well characterized, the exact initiating events that lead to failure of immunoregulation and to autoimmunity are not understood (Al-Ghazlat, 2009).

Current therapy for IMHA consists of supportive care and non-specific suppression of the immune response (Grundy & Barton, 2001; Mason et al, 2003; Scott-Montcrieff et al, 2001; Weinkle et al, 2005; Piek et al, 2008). Treatment can therefore be thought of as being largely palliative (i.e., controlling signs of disease), rather than curative (i.e., addressing the mechanisms underlying immune dysregulation). Moreover, current immunosuppressive strategies are associated with certain risks: for example, immunosuppressive doses of glucocorticoids - the mainstay of IMHA treatment - predispose the patient to a variety of serious and potentially fatal complications, including severe infection and thromboembolic disease (Carr et al, 2002; Scott-Montcrieff et al, 2001; Semple & Freedman, 2005; Weinkle et al, 2005). Regardless of treatment, at present, canine primary IMHA carries a high mortality rate, ranging from 23% to 70%, with death usually occurring within the first 2 to 3 weeks of treatment (Grundy & Barton, 2001; Mason et al, 2003; Scott-Montcrieff et al, 2001; Weinkle et al, 2005; Piek et al,
Clearly, more effective therapies, which specifically target dysfunctional immunoregulatory mechanisms without causing nonspecific immune suppression and unwanted adverse effects, are needed.

As studies in human and murine models of autoimmunity demonstrate (e.g. multiple sclerosis, autoimmune anterior uveitis, experimental autoimmune encephalomyelitis and type I diabetes), identification of target autoantigens is an important step towards understanding mechanisms which underlie breakdown of self-tolerance, and is an avenue of research critically lacking in canine IMHA. Additionally, knowledge of dominant autoantigens can lead to the development of targeted immunotherapies (Shen et al, 2002; Faria & Weiner, 2006; Fontura et al, 2005). A comprehensive search of the medical and veterinary databases as far back as two decades revealed only two studies that attempted to determine target antigens in canine primary IMHA: Barker and colleagues (Barker et al, 1991; Barker & Elson, 1995) found that a subset of dogs with primary IMHA (4 out of 7 in the first study, and 2 out of 4 in the second study) had antibodies specific for glycophorins, and therefore the latter are likely a target autoantigen in some dogs with IMHA. However, both studies were hampered by small sample size, and there was no attempt to assess reactivity with other RBC membrane proteins in the patients that lacked apparent reactivity against glycophorins.

The goals of the present study were (1) to characterize RBC membrane proteins from a pool of healthy dogs using one-dimensional SDS-PAGE; and (2) to directly identify erythrocyte membrane antigens that are targeted by the immune system in dogs diagnosed with primary IMHA.
Materials and Methods

Criteria for Selection

**Primary IMHA dogs.** Dogs with acute-onset IMHA as defined by clinical signs <1 week in duration and PCV <25% that presented to the Ontario Veterinary College Teaching Hospital (OVCTH) were eligible for inclusion in this study. Cases were collected between the period of April 2007 and August 2008. Primary IMHA was diagnosed on the basis of history, compatible clinical signs (e.g. lethargy, exercise intolerance), the presence of anemia with persistent saline agglutination, slide agglutination or positive direct Coombs’ test, spherocytosis, or evidence of hemolysis (i.e. hyperbilirubinemia, hemoglobinemia, or hemoglobinuria). Patients in whom an underlying potential cause (e.g. positive titer for infectious disease, neoplasia) of IMHA was identified by history, physical examination, or laboratory tests were excluded. Also excluded from the study were dogs that received glucocorticoids >3 days, ≥1 dose of other immunosuppressant drugs (e.g. azathioprine, cyclophosphamide, or cyclosporine), or other drugs (excluding anthelmintics/heartworm preventatives) prior to onset of clinical signs.

**Anemic, non-IMHA dogs.** Dogs with causes of anemia (regenerative or non-regenerative) other than IMHA, admitted to the OVCTH between April 2007 and August 2008, were also included in the study. IMHA was ruled out based on the absence of microscopic slide or saline agglutination, lack of spherocytosis, and the presence of an underlying condition explaining the anemia, based on general physical examination, history, and ancillary tests (e.g. ultrasonography, histopathology, etc.). Dogs that had received previous blood transfusions were excluded.
**Control dogs.** Control dogs were selected from a pool of blood donor dogs at the OVCTH and adult dogs belonging to faculty, students and staff at the Ontario Veterinary College during the period of April, 2007 to August, 2008. Dogs were at least 1 year of age, ≥25 kg, and deemed healthy based on history and general physical examination. Dogs did not have a history of travel outside of Canada, previous blood transfusions, pregnancy (if female), current serious or chronic diseases, drug administration (other than anthelmintics/heartworm preventative) <4 weeks prior to inclusion, vaccinations <3 weeks prior to sample collection, and had negative serology for heartworm and tick-borne diseases.

**Initial Data on Presentation**

A complete history documenting travel, past and current medical conditions, current medications, toxin or drug exposure, clinical signs and duration of illness prior to presentation was taken by the attending veterinarian on admission to OVCTH. A physical examination was also performed. Initial blood work included serum chemistry (Hitachi 917, Roche Diagnostics, Toronto, ON, Canada) and automated complete blood count (Advia 120, Siemens, Mississauga, ON, Canada) with manual differential. A direct Coombs’ test was ordered only if IMHA was suspected and microscopic agglutination was not obvious during blood smear examination or on saline agglutination test.

**Sample Collection and Processing**

**Dogs with primary IMHA (Patient dogs, n=15) and non-IMHA anemic dogs (n=4).** EDTA anticoagulated blood from each dog was collected upon admission to OVCTH, prior to treatment, and stored for no more than 48 hours at 4°C Celsius until processing. The blood was centrifuged at 1000x g for 5 minutes; supernatant plasma was
removed and stored in 500 uL aliquots at -70°Celsius. Buffy coat was discarded. Red cells were saved for further processing.

**Control dogs (n=17).** EDTA anticoagulated from each control dog was collected and stored at 4°Celsius for no more than 4 hours, until processing. RBCs from this cohort of dogs were used for the following: (1) to generate pooled RBC lysates; and (2) if dog erythrocyte antigen (DEA) 1.1 positive, to serve as positive controls validating the xylene elution technique.

**Elution of RBC-bound antibodies**

Several techniques to elute RBC-bound antibodies have been described. We chose to use a xylene elution technique as described by Chan-Shu and Blair (1979), which is based on disruption of van der Waals interactions between antigen and antibody by the addition of excess amounts of organic solvent (Judd, 1993). Other organic solvents such as ether and dichloromethane have been used successfully (all based on the same chemical principle of disrupting antigen-antibody interactions). However, South and colleagues (1985) evaluated eleven different red cell elution procedures on red cells sensitized with known amount of immunoglobulin and demonstrated that in terms of antibody recovery xylene proved to be most effective.

Xylene eluates of RBC were obtained from patient dogs, non-IMHA dogs, and control dogs. Packed red cells were washed in EDTA-phosphate buffered saline (EDTA-PBS, pH 7.4), at a 1:4 ratio of red cells-to-saline, 5-7 times. After the final wash, EDTA-PBS equal in volume to red cells was added to the tube and mixed well by gentle inversion. Two volumes of xylene were added to the tube, which was subjected to gentle inversion to mix contents, and then incubated in a 56°Celsius water bath for 10 minutes. After incubation, the mixture was centrifuged at 1000 x g for 10 minutes. Three distinct
layers were observed following centrifugation: an upper layer of xylene, a middle
“stromal” layer (comprised of RBC membranes), and hemoglobin-stained eluate on the
down. The upper two layers were vacuum aspirated and discarded. The bottom eluate
layer was recovered, and stored at -70°C Celsius in 250 uL aliquots, until further use.

Sensitization of RBC – Positive control samples for xylene elution technique

RBCs from Dog Erythrocyte Antigen (DEA) 1.1-positive dogs (selected from the
blood donor program at OVCTH, typed 3 or greater reaction based on the ID-Gel Test,
DiaMed-VET, Morat, Switzerland) were separated from plasma, and then washed at least
5 times in EDTA-PBS. While RBCs were being washed, lyophilized canine antiserum
(Animal Health Laboratory, Guelph, ON, Canada) containing antibodies against DEA 1.1
was reconstituted as described in the Standard Operating Procedures of the Animal Health
Laboratory at the University of Guelph (AHL): to each vial of 100 mg of lyophilized
antisera, 1.0 mL of Milli-Q water was added and then mixed for a few seconds using a
vortex mixer. Lyophilized canine antiserum was generated by allogeneic blood
transfusion of DEA 1.1 red cells in DEA 1.1 negative dogs. An equal volume of the re-
constituted antiserum was added to the tube of washed, DEA 1.1 RBCs. The tube was
sealed and incubated in a 37°C water bath for 2 hours. After incubation, the tube was
centrifuged at 800 x g for 5 minutes, and the supernatant antiserum was discarded.
Sensitized RBCs were then washed 3-5 times in physiologic saline to remove any excess
or non-specifically bound antibody (i.e. until supernatant was clear). The xylene elution
technique (Chan-Shu & Blair, 1979) was then applied to these washed, sensitized RBCs,
and eluate stored in 250 uL aliquots at -70°C Celsius until use.
**RBC lysate generation**

RBC lysates were generated from packed RBCs of healthy control dogs, in a manner previously described by Doberstein et al (1995). Briefly, packed RBCs obtained during initial sample collection were washed in approximately ten volumes of ice cold EDTA-PBS at least 4 times (each wash involved centrifugation at 800 x g for 5 minutes at 4°C). Next, forty volumes of hypotonic saline (5 mM NaPO₄, pH 7.5) were added to one volume of washed RBC pellet. One milliliter of 100 mM phenylmethylsulfonylfluoride (PMSF) was added to each 100 mL of hypotonic saline prior to addition of hypotonic saline to the RBC pellet. The entire suspension was stored on ice for 10 minutes to allow complete lysis of RBCs, and then centrifuged on a Sorvall RC-5B centrifuge at 22,610 x g for 15 minutes at 4°C. The supernatant was removed and discarded, and the wash step using ice-cold hypotonic saline with PMSF was repeated (omitting the incubation phase) until the supernatant was clear, and a white pellet was observable on the bottom of the centrifuge tube. After final pelleting, the lysates were re-suspended in 10 volumes of hypotonic saline, and stored in 500 uL aliquots at -20°C Celsius for no more than 4 months (each aliquot contained 50 uL of pelleted lysate plus 450 uL of hypotonic saline).

**One-dimensional (1D) SDS-PAGE**

**RBC membrane proteins (from lysates).** One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) of RBC membrane proteins was performed, based on the technique first described by Laemmli (1970; Gallagher, 2000). SDS-PAGE buffer solutions, including electrophoresis buffer and those used for casting gels, were reconstituted from commercially available, pre-made stock solutions (Bio-Rad Corp., Mississauga, ON, Canada), and electrophoresis was performed using the
Bio-Rad modular Mini-Protean II system (Bio-Rad, Mississauga, ON, Canada). Erythrocyte lysates were allowed to thaw out at room temperature just prior to use. Lysate solution was mixed with 6x SDS sample buffer in a 1:1 ratio, and incubated in a 65°C Celsius water bath for 10 minutes. A ten microliter aliquot of this sample mixture plus sample buffer was then loaded into each sample well of a 1.0 mm thick, 15% Tris-HCl polyacrylamide gel. The gel was run at 250 V constant for 30 minutes. Pre-stained broad range molecular weight markers were used to identify apparent molecular weights on the gel (Dual Color Precision Plus Protein Standards, Bio-Rad, Mississauga, ON, Canada). Gels were stained with a mass spectrometry-compatible silver nitrate protocol described by Shevchenko and others (Shevchenko et al, 1996).

**RBC eluates.** 1D SDS-PAGE of RBC eluates was also performed as described above, with the following differences. Before use, aliquots of eluate were thawed at room temperature, and then centrifuged at 5,000 x g for 5 minutes to remove any contaminant stroma. Ten microliters of sample buffer plus eluate were loaded in each well of a 1.0 mm thick gel with final acrylamide concentration of 15% (w/v), and run at 225 V constant for 55 minutes.

**Blue Native PAGE**

Blue native PAGE of RBC membrane proteins was performed as previously described (Reisinger and Eichacker, 2006; Wittig et al., 2006). This procedure was carried out to achieve high resolution non-denaturing separation of membrane integral proteins and protein complexes. Aliquots of RBC lysate were thawed out at room temperature just prior to use. Each 500 uL aliquot was centrifuged at 16,000 x g for 5 minutes, and supernatant (hypotonic saline) was discarded. To the remaining pellet (50 uL total), 37 uL of digitonin solubilization buffer was added and thoroughly mixed, and then incubated on
ice for 1 hour. Finally, the mixture was centrifuged at 16,700 x g for 10 minutes. Twenty-five microliters of supernatant, which contained solubilized membrane proteins, were placed in a fresh 250 μL cap, to which 2.5 μL of loading buffer was immediately added. Eighteen microliters of sample mixture plus loading buffer were loaded into each sample well of a 5-14% gradient gel, and electrophoresis was performed on ice, as previously described. Running conditions were as follows: 250 V constant for 30 minutes using the blue cathode running buffer (which was carefully discarded after that time), then 225 V constant in the final 1 hour and 30 minutes, using the clear cathode running buffer (anode buffer remained the same throughout electrophoresis). Gels were stained with a mass spectrometry-compatible silver nitrate protocol described by Shevchenko et al. (1996) (Appendix 1).

**Immunoblot (western blot) Analysis**

**Positive control eluates.** In order to validate the presence of immunoglobulin in xylene eluates, electroblots of gels using positive control eluates (DEA 1.1-positive, sensitized RBCs) were performed and compared with electroblots of purified canine IgG (VMRD Inc., Pullman, WA, USA). 1D SDS-PAGE gels of positive control eluate and those of purified canine IgG were transferred onto nitrocellulose as described in Ursitti et al. (2000). Electroblots were blocked with 1% bovine serum albumin in Tris buffered saline (BSA-TBS), washed in TBS with 1% Tween 20 (TBS-T) for 3 times (15 minutes each), and then incubated with 1:5000 polyclonal sheep anti-canine IgG (antibody AA132P, AbD Serotec, Oxford, UK) conjugated to horseradish peroxidase for 1 hour at room temperature. The secondary antibody was manufactured by repeated immunization of sheep with highly purified antigen (canine IgG). Sheep IgG was then purified by
affinity chromatography. According to the manufacturer, there is no cross reactivity of this antibody against other classes of canine immunoglobulin.

Following a second round of washing with TBS-T, the blots were incubated with chemiluminescent detection system for HRP as recommended by the manufacturer (ECL, GE Healthcare, Baie d’Urfe, QC, Canada), and then developed on x-ray film (Hyperfilm ECL, GE Healthcare, Baie d’Urfe, QC, Canada). All antibodies were diluted with 1% BSA-TBS.

**Transfer of BN-PAGE gels.** BN-PAGE gels of DEA 1.1-positive RBC lysates were transferred onto nitrocellulose as described previously (Ursitti et al, 2000). Due to the large size of the proteins, transfer was performed for 2 hours and 30 minutes at 200 mA constant current on ice. Successful transfer of proteins was confirmed by Ponceau S membrane staining (BioRad, Mississauga, ON) followed by de-staining with Milli-Q water. The blot was blocked in 1%-BSA-TBS and washed in TBS-T as previously described. Eluates made from sensitized DEA 1.1-positive RBCs were used as primary antibody, followed by 1:5000 sheep anti-canine IgG conjugated to HRP.

**RBC lysate as target.** 1D SDS-PAGE gels of RBC lysate were transferred to nitrocellulose as previously described (Ursitti et al, 2000). The blots were then blocked in 1% BSA-TBS, washed 3 x 20 minutes in TBS-T, and then incubated with 1:75 dilution of RBC eluate, which served as primary antibody, at room temperature for 90 minutes. After washing again with TBS-T, the blots were incubated in 1:5000 dilution of sheep anti-canine IgG conjugated to HRP for 1 hour. A third wash cycle was followed by incubation with chemiluminescent detection system for HRP, and the blots were developed on x-ray film as above.
**RBC eluate as target.** Proteins in xylene eluates were also separated by electrophoresis, transferred to membranes, and probed for canine Ig, as described above.

**Identification of Proteins**

**Sample processing.** For each immunoblot analysis, two SDS-PAGE gels were prepared simultaneously: one gel was used for electroblot transfer, while the other was stained using a mass spectrometry-compatible silver nitrate protocol (Shevchenko et al., 1996). SDS-PAGE bands which corresponded to bands on immunoblots (based on relative position) were manually excised, placed in a siliconized cap filled with 1% v/v acetic acid, and sent to The Advanced Protein Technology Centre, Hospital for Sick Children (Toronto, ON, Canada) for analysis. In-gel tryptic digests were analyzed by mass spectrometry. Peptide sequencing was determined by on-line liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA) coupled to an Agilent 1100 Capillary LC system (Santa Clara, CA, USA). Sample (5 uL) was loaded into a pre-column (100 um internal diameter x 5 cm length), and then eluted to an analytical column (75 um i.d. x 10 cm length), both of which were packed with Magic C18 resin (Michrom Bioresources, Auburn, CA, USA) for further separation. For reverse phase chromatography, a gradient elution from water to acetonitrile, each containing 0.1% formic acid and 0.02% trifluoroacetic acid, was carried out over 100 minutes. Tandem mass spectra were extracted and analyzed using Mascot (Matrix Science, London UK) and X!Tandem (http://www.thegpm.org; Beavis Informatics, Winnipeg, AB, Canada) using the NCBI protein sequence database (RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq/). The database was searched for proteins matching mass spectra within the taxonomic category *Canis familiaris*, but if no matches were
found, further searches were performed to include all mammalian species. Scaffold (Proteome Software Inc., Portland, OR, USA, version 2.1.0) was used to validate tandem MS-based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95% probability and included at least two identified peptides.

**Results**

**Primary IMHA patient cases**

Seventeen (17) cases with an initial diagnosis of primary IMHA were collected during the period of April 2007 and August 2008. One case (case 6) was subsequently determined to have splenic torsion, and was included instead in the non-IMHA, anemic group. Case 8 was later excluded because it was revealed in subsequent communications with the regular veterinarian that the dog had received a whole blood transfusion prior to referral to OVCTH. Case 17 was later found to have metastatic lung carcinoma on necropsy; and insufficient sample was collected from case 16 due to the small size of the patient and severe anemia. This left a total of thirteen (13) cases that met the inclusion criteria for the study. The cases consisted of 8 females (8/13, 62%) and 5 males (5/13, 38%), ranging in age from 3 to 12 years (mean ± SD, 7.2 ± 3.3 years). Hematocrit on admission to OVCTH ranged from 6% to 18% (mean ± SD, 12.2 ± 4.2%). Breeds included Border collie (2/13), Golden Retriever (2/13), Cocker Spaniel (2/13), mixed breed (2/13) and one each of Dachshund, Bichon Frise, rough-coated collie, Australian cattle dog, and Whippet (see Table 1).
Table 1: Clinical and laboratory findings in dogs with IMHA

<table>
<thead>
<tr>
<th>No.</th>
<th>Age(^a) (yrs)</th>
<th>Signalment</th>
<th>Hct(^b) (L/L)</th>
<th>History and clinical signs</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>FS(^c) Border Collie</td>
<td>0.15</td>
<td>Lethargy, anorexia, icterus, tachycardia, systolic heart murmur</td>
<td>Euthanized</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>FS Dachshund</td>
<td>0.10</td>
<td>Lethargy, anorexia, tachycardia, pale mucous membranes, lymphadenomegaly</td>
<td>Alive 6 months after referral</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>FS Border Collie</td>
<td>0.06</td>
<td>Lethargy, inappetance, hematuria, pale mucous membranes</td>
<td>Euthanized</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>FS Bichon Frise</td>
<td>0.17</td>
<td>Lethargy, inappetance, soft stools, icterus, tachycardia</td>
<td>Alive 1 month after referral; lost to follow-up</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>FS Rough Coated Collie</td>
<td>0.12</td>
<td>Lethargy, inappetance, icterus, tachycardia</td>
<td>Alive 1 month after referral; lost to follow-up</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>FS Australian Cattle dog</td>
<td>0.17</td>
<td>Lethargy, anorexia, vomiting, hematuria, fever, tachycardia</td>
<td>Discharged, sudden death at home 3 months later</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>MC(^d) Golden Retriever</td>
<td>0.12</td>
<td>Lethargy, anorexia, icterus, tachycardia, heart murmur</td>
<td>Alive 1 year after initial referral</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>FS Mixed Breed</td>
<td>0.08</td>
<td>Vomiting, anorexia, lethargy, heart murmur, tachycardia, pale mucous membranes</td>
<td>Alive 4 months after initial referral</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>MC Cocker Spaniel</td>
<td>0.16</td>
<td>Lethargy, anorexia, hematuria and icterus</td>
<td>Euthanized</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>MC Whippet</td>
<td>0.18</td>
<td>Lethargy, anorexia, vomiting, heart murmur, pale mucous membranes</td>
<td>Alive 2 months after initial referral</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>MC Golden Retriever</td>
<td>0.09</td>
<td>Lethargy, anorexia, tachycardia, pale mucous membranes</td>
<td>Alive 8 months after initial referral</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>MC Cocker Spaniel</td>
<td>0.06</td>
<td>Lethargy, anorexia, hematuria, vomiting, tachycardia</td>
<td>Alive 8 months after initial referral</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>FS Mixed Breed</td>
<td>0.13</td>
<td>Lethargy, excessive panting, anorexia, tachycardia, pale mucous membranes</td>
<td>Alive 8 months after initial referral</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SD = 7.2 ± 3.3  
\(^b\)Hct = hematocrit, mean ± SD = 0.122 ± 0.042  
\(^c\)FS = female spayed  
\(^d\)MC = male castrated
Case 1 was a female-spayed Border collie who was 3 years old at the time of initial presentation to the referring veterinarian. She presented because of acute onset severe lethargy and anorexia of about 48 hours duration. She was referred to OVCTH within a few hours of presenting to the veterinarian. There was no history of travel outside of Ontario, serious illnesses, or recent medications (other than heartworm preventatives of unknown type) relayed to the referring veterinarian or to the attending veterinarian at OVCTH by the dog’s owners. On initial physical examination at OVCTH, she had pale but icteric mucous membranes, tachycardia (HR = 120 beats per minute), and grade III/VI systolic murmur with point of maximal intensity (PMI) on the left apex. Packed cell volume (PCV) on presentation was 15% and total protein was 70 g/L (55-74 g/L). There was both marked slide agglutination and microscopic autoagglutination, 3 spherocytosis, and >12-15 polychromatophils per 100 x objective. Plasma was markedly icteric. The dog was also hypokalemic (2.6 mmol/L; reference interval 3.5-4.9 mmol/L) based on whole blood analysis on i-STAT point-of-care analyzer (Abbott, Mississauga, ON, Canada), but remaining analytes were within reference intervals. Given the guarded prognosis, potential for intensive medical care and financial constraints of the owners, the dog was humanely euthanized that day.

On necropsy examination, the dog was found to be in good body condition, although the subcutaneous and adipose tissues were markedly jaundiced. The liver was firm and congested, but no other gross abnormalities were noted. Histopathology revealed mild pulmonary edema, as well as increased numbers of hemosiderin-laden macrophages throughout the liver and red pulp of the spleen, which was attributed to acute hemolytic disease. No other histopathologic abnormalities were noted.
Case 2 was a female-spayed Dachshund who presented to her regular veterinarian because of acute onset listlessness and anorexia of approximately 1-2 days duration. At the time of presentation, the dog was 10 years of age. She had not traveled outside of Ontario, was not currently on medications other than heartworm preventative, nor did she have a history of serious illnesses. The dog’s veterinarian noted tachycardia, white mucous membranes, and mild peripheral lymphadenomegaly on physical examination. In-house blood work revealed marked anemia (PCV = 13%) with normal total solids and positive slide agglutination. The veterinarian administered a total of 2.0 mg dexamethasone IM and 0.4 mL enrofloxacin (Bayer, Shawnee Mission, KS, USA), and sent the dog home with instructions to return for re-check. The following day, PCV declined to 10% and the dog was reported to be clinically worse, so she was referred immediately to OVCTH. General physical examination on presentation at OVCTH revealed tachycardia (HR = 200 with bounding pulses) and pale icteric mucous membranes, but was otherwise unremarkable. CBC/differential performed on admission at OVCTH confirmed the presence of severe anemia (10%) that was also macrocytic (MCV = 84 fL; 62-72 fL), hypochromic (MCHC = 299 g/L; 330-360 g/L), and markedly regenerative (absolute reticulocyte count = 556 x 10⁹/L; <80 x 10⁹/L). There was a mild leukocytosis (WBC 16.0 x 10⁹/L; 4.9 – 15.4 x 10⁹/L) composed of neutrophilic left shift with mild toxicity and monocytosis, which are changes consistent with inflammation. Serum chemistry was unremarkable except for the presence of increased total bilirubin concentration (6 umol/L; 0-4 umol/L) and increased alkaline phosphatase activity (375 U/L; 22 – 143 U/L), which were referable to hemolysis, cholestasis, drug induction or a combination thereof. Results of total body imaging (chest radiographs and abdominal ultrasound) were unremarkable, and serology for tick-borne diseases (including
borreliosis, anaplasmosis and ehrlichiosis) was negative. The dog was started on a course of immunosuppressive therapy (dexamethasone 0.25 mg/kg IV q24h; azathioprine 2 mg/kg PO q24h), aspirin 0.5 mg/kg PO q24h, famotidine 0.5 mg/kg IV q12h), and intravenous fluids (Plasmalyte A, Baxter, Mississauga, ON, Canada). Due to unstable PCV, she was given a total of 2 packed RBC transfusions within the first 48 hours of hospitalization. By the fourth day, her demeanor had improved and her PCV was stable at 23%. She was discharged on a course of oral immunosuppressive drugs, gastrointestinal protectants, and aspirin. The dog returned to OVCTH for follow-up, and at her last visit (six months after initial presentation), her PCV was 42% and she was clinically normal.

Case 3 was a female spayed Border collie who was 12 years of age at the time of initial presentation to her regular veterinarian. She was initially presented because of lethargy and “red urine.” The veterinarian prescribed trimethoprim-sulfa for suspected urinary tract infection (dose unknown), but the dog rapidly deteriorated in the next 24 hours, necessitating a visit to the local veterinary emergency clinic the next day. Blood work performed at the emergency clinic revealed a moderate leukocytosis, severe anemia (HCT = 7%), hyperbilirubinemia, and increased alkaline phosphatase and alanine aminotransferase activities. The dog was referred immediately to OVCTH. CBC on presentation to OVCTH confirmed a severe anemia (HCT = 6%; 39-56%) that was macrocytic (MCV = 112 fL; 62-72 fL) and strongly regenerative (313 x 10^9/L; <80 x 10^9/L). A moderate rubricytosis accompanied the regenerative response. There was also marked autoagglutination, spherocytosis, and many red cell ghosts, findings indicative of both extravascular and intravascular hemolysis. Moderate leukocytosis (corrected WBC = 36.2 x 10^9/L; 4.9-15.4 x 10^9/L) was composed of moderate neutrophilia with left shift and mild toxic changes, and monocytosis, which were changes attributed to inflammation.
Serum chemistry showed the following abnormalities: hyperphosphatemia (3.11 mmol/L; 0.90-1.85 mmol/L) and moderate azotemia (urea = 23.6 mmol/L; creatinine = 190 umol/L), which were referable to dehydration and decreased glomerular filtration rate (GFR); marked hyperbilirubinemia (total bilirubin was 51 umol/L; 0-4 umol/L) and increased alkaline phosphatase activity (860 U/L), which were consistent with hemolysis, cholestasis, steroid induction of alkaline phosphatase, or a combination thereof; increased alanine aminotransferase activity (2658 U/L; 19-107 U/L), which indicated hepatocellular damage, most likely from hypoxia secondary to severe anemia; and mild to moderately elevated amylase (1595 U/L; 299-947 U/L) and lipase (9020 U/L; 60-848 U/L) activities, which were attributed to a combination of glucocorticoid induction, pancreatitis, and decreased renal elimination. A course of intravenous dexamethasone (0.25 mg/kg q24h), low-dose aspirin, and azathioprine as well as intravenous fluids (Plasmalyte A) were commenced (dosages as described previously), and the dog was giving a packed RBC transfusion. Initial therapy improved PCV to 22%, but the dog developed profuse vomiting, which was suspected to be an adverse reaction to the azathioprine (likely acute pancreatitis). Azathioprine was discontinued, and cyclosporine (5 mg/kg PO q24h) was begun. Although the vomiting abated, the dog continued to decline clinically despite 2 further blood transfusions. Coagulation panel four days after admission showed markedly prolonged prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrin degradation products (FDP), indicating disseminated intravascular coagulation. The owners elected humane euthanasia.

Case 4 was a female spayed Bichon Frise that was 11 years old at the time she was initially presented to the regular veterinarian. The dog had a history of inappetance, lethargy, and soft stools of approximately 1-2 days duration. A PCV revealed moderate
anemia (25%) with normal total solids and positive slide agglutination. In-house lab work also showed mildly elevated amylase (2077 U/L; 190-1350 U/L). The regular veterinarian started the dog on a course of prednisone (10 mg PO q12h), and discharged her with the instructions to recheck the following day. Over the course of the next 48 hours, the PCV had decreased to 17%, (but total solids were stable at 73 g/L), and the owner was having difficulty administering the prednisone, so the dog was referred to OVCTH. Initial physical examination at OVCTH was unremarkable except for extreme pallor of mucous membranes and tachycardia (HR = 200). CBC confirmed the presence of a severe, macrocytic, hypochromic and markedly regenerative anemia (HCT = 17%; MCV = 79 fL; MCHC = 300 g/L; absolute reticulocytes = 561.7 x 10⁹/L) that was accompanied with moderate spherocytosis, autoagglutination, and an appropriate rubricytosis. Mild leukocytosis (16.4 x 10⁹/L) was composed of moderate neutrophilia, left shift with mild toxicity, lymphopenia (0.33 x 10⁹/L; 0.8-5.1 x 10⁹/L) and monocytosis (2.30 x 10⁹/L). The leukogram was consistent with inflammation and cortisol response. Serum chemistry profile revealed mildly increased urea (10.3 mmol/L; 3.5-9.0 mmol/L) with normal creatinine, which indicated early decrease in GFR and/or gastrointestinal hemorrhage. The mildly decreased total protein (51 g/L; 55-74 g/L) composed of mildly decreased albumin (28 g/L; 29-43 g/L) and low-normal globulins (23 g/L; 21-42 g/L) was consistent with gastrointestinal (GI) hemorrhage (protein loss), although other factors such as inflammation (albumin as negative acute phase reactant) and immunoglobulin production related to immune-mediated disease could also explain these changes.

Results of full work-up, including imaging and serology for tick-borne diseases, were within normal limits, and the dog was started on a course of immunosuppressive therapy (dexamethasone 0.25 mg/kg IV q24h, cyclosporine 5 mg/kg POq24h, and
azathioprine, 1.5 mg/kg PO q24h), aspirin (0.5 mg/kg PO q24), famotidine (0.5 mg/kg IV q12h) and intravenous fluids (Plasmalyte A, Baxter, Mississauga, ON, Canada). Over the course of 4 days, the dog’s condition stabilized and she was discharged. One month after discharge, her PCV was 41% and she was clinically normal, but she was lost to follow-up after that time.

**Case 5** was a female spayed rough-coated collie that was 6 years old at the time of initial presentation to the regular veterinarian. Her owner had noted progressive lethargy and inappetance for at least 3 days duration. The dog did not have a history of travel outside of Southern Ontario, she was well supervised with no previous serious illnesses or medications other than heartworm preventatives, and her vaccinations were up-to-date (her last one over 6 months prior to presentation). Results of general physical examination by the regular veterinarian were unremarkable except for pale icteric mucous membranes. In-house lab work showed moderate anemia (HCT = 19%; 39-60%), a mild mature neutrophilia (12.2 x 10^9/L; 2.5-11.2 x 10^9/L), and marked hyperbilirubinemia (56.8 umol/L; 0-5 umol/L). Due to the suspicion of IMHA, the dog was referred to OVCTH. Initial physical examination results at OVCTH were unremarkable other than pale icteric membranes and mild tachycardia (HR = 120 bpm). CBC revealed marked anemia of 12%, with marked autoagglutination, spherocytosis and RBC ghosts, consistent with extravascular and intravascular hemolysis. The plasma was grossly hemolyzed, thus elevated MCHC (442 g/L; 330-360 g/L) was probably artifact and attributable to the hemolysis. Absolute reticulocyte count (200 x 10^9/L) indicated an appropriate regenerative response. Mild leukocytosis (22.9 x 10^9/L) was composed of mild neutrophilia with left shift and mild to moderate toxicity, which was consistent with inflammation.
Abnormalities in serum chemistry profile included the following: decreased total CO₂, which is indicative of metabolic acidosis; mild hypokalemia (3.5 mmol/L; 3.8-5.4 mmol/L) attributed to anorexia and transcellular shifting from acidosis; increased urea (15.2 mmol/L) with normal creatinine, which was due probably to gastrointestinal hemorrhage; and marked hyperbilirubinemia (total bilirubin 90 umol/L; 0-4 umol/L), which was attributed to hemolysis.

A thorough work-up, including serology for tick-borne diseases and imaging, failed to disclose an underlying cause of anemia, and the dog was begun on a course of immunosuppressive drugs (including azathioprine and dexamethasone at dosages previously described), intravenous fluid therapy (Plasmalyte A, Baxter, Mississauga, ON, Canada) and gastrointestinal protectants as previously described. Over the course of the next week, PCV continued to decline and the dog received a total of 3 packed RBC transfusions. Finally, 10 days after admission to the OVCTH, the dog’s PCV stabilized at 28% and her clinical condition improved enough for her to be discharged to the care of her regular veterinarian on an outpatient basis. Roughly one month after discharge, the regular veterinarian contacted OVCTH to report that the dog was clinically doing well, and that her PCV was 32%.

**Case 6** was a 5 year-old male castrated Weimeraner who was initially presented for suspected IMHA, but was subsequently determined to have a splenic torsion. His case is included with the other non-IMHA anemic dogs.

**Case 7** was a female spayed, Australian cattle dog 3 years old at the time of initial presentation. Her owners noted to their regular veterinarian that the dog had been lethargic, vomiting and had “red urine” for the past 1-2 days. The dog was well supervised, with no history of travel outside of Ontario, no previous serious health
concerns or current medications other than heartworm preventative. She was current on vaccines. Physical examination by the regular veterinarian revealed a fever of 40.2\(^\circ\) Celsius and mild tachycardia (HR = 160 bpm). In house lab work by the regular veterinarian showed moderate anemia of 28% (37-55%), with an absolute reticulocyte count of 109.7 x 10\(^9\)/L and positive slide agglutination. The dog was referred directly to OVCTH. CBC was repeated at OVCTH and confirmed the presence of moderate to severe, regenerative anemia and a leukogram consistent with inflammation: HCT 17% (39-56%); absolute reticulocyte count of 264.8 x 10\(^9\)/L (<80 x 10\(^9\)/L); corrected WBC 26.8 x 10\(^9\)/L (4.9-15.4 x 10\(^9\)/L); neutrophil count 20.1 x 10\(^9\)/L (2.9-10.6 x 10\(^9\)/L); band neutrophil count 0.54 x 10\(^9\)/L (0.0-0.3 x 10\(^9\)/L); monocyte count 2.14 x 10\(^9\)/L (0.0 – 1.1 x 10\(^9\)/L); rubricyte count 1.34 x 10\(^9\)/L. Plasma was grossly hemolyzed, and examination of the blood smear disclosed the presence of spherocytes, autoagglutination and ghost RBCs. Results of serology for tick-borne diseases and heartworm were negative, and abdominal ultrasound failed to reveal any abnormalities. The dog was treated with a course of immunosuppressive drugs (azathioprine and dexamethasone), aspirin, and famotidine at dosages previously described. After 8 days, her PCV had stabilized to 21% and clinical condition improved significantly, and she was discharged with instructions to continue oral immunosuppressant drugs and follow up regularly with her veterinarian. Roughly 3 months after discharge, her veterinarian contacted OVCTH with the news that the dog was doing very well clinically, and had a PCV of 41%. By this time, she had been weaned off prednisone and was only on azathioprine at a much reduced dosage. However, one month after this communication, the dog died suddenly at home, with apparently no prodromal illness. A necropsy was not performed.
Case 9 was a male castrated, 4-year old Golden Retriever who was presented to his regular veterinarian because of acute onset lethargy and anorexia of 1 day duration. There was no history of travel outside of Ontario, although the owners did have a cabin in Northern Ontario which they visited frequently with the dog. The dog had otherwise been in good health, and was not on medications other than heartworm preventative. Results of physical examination by the regular veterinarian included tachycardia (HR = 160 beats per minute), pale icteric mucous membranes, and a grade II/VI systolic murmur that had been present since the time of birth. PCV performed at the referring clinic revealed a severe anemia of 12%. Additionally, in-house lab work showed severe hyperbilirubinemia (43.1 umol/L; 0-15 umol/L). The dog was sent immediately to OVCTH. Results of general physical examination at OVCTH were consistent with those obtained earlier by the referring veterinarian. CBC on admission showed severe macrocytic, poorly-regenerative anemia (HCT = 12%; absolute reticulocyte count 61.7 x 10^9/L) that was accompanied by marked autoagglutination and occasional RBC ghosts. Spherocytes were difficult to see due to the severity of agglutination. There was a mild leukocytosis (22.8 x 10^9/L), consisting of mild neutrophilia with left shift (segmented neutrophils 18.47 x 10^9/L; band neutrophils 1.39 x 10^9/L), indicative of inflammation. Additionally, hyperbilirubinemia due to severe, acute hemolysis was also confirmed (total bilirubin = 46 umol/L; 0-4 umol/L). The lack of a robust regenerative response at the time of admission was attributed to the acute onset of illness, and the rapidity with which the owners sought veterinary care. Repeat CBC and reticulocyte count two days later showed improving HCT (17%) and adequate regenerative response (absolute reticulocyte count = 269.7 x 10^9/L). Full work-up, including tick-borne disease and heartworm serology, abdominal and thoracic imaging, failed to reveal an underlying cause of the IMHA.
The dog was treated with a course of immunosuppressive drugs (dexamethasone and azathioprine), famotidine, aspirin and intravenous fluids at dosages described previously. After a total of 2 packed RBC transfusions and 7 days in hospital, he was discharged. Follow-up visits at the regular veterinary clinic and at OVCTH found the dog to be improving over the ensuing months; two months after discharge, his PCV was stable at 37%. However, six months after initial presentation, the dog developed iatrogenic hyperadrenocorticism (as determined by compatible clinical signs) and a urinary tract infection (which was treated with antibiotics, dose and type unknown). Prednisone dose was slowly tapered and the hyperadrenocorticism resolved within several months. The dog continued on oral azathioprine, but was lost to follow-up one year after discharge.

Case 10 was a female spayed, 7-year-old mixed breed dog who was presented to the regular veterinarian with a 2 day history of gagging and vomiting, anorexia, and severe lethargy. The dog had previously been healthy, other than a urinary tract infection that was treated with antibiotics (type and dose unknown) 6 months prior. CBC and serum chemistry performed at the regular veterinary clinic showed the following abnormalities: HCT 19% (39-60%); segmented neutrophils 17.3 x 10^9/L (3-11.5 x 10^9/L); total bilirubin 47.7 umol/L (0-5 umol/L); and increased alanine aminotransferase activity 156 U/L (15-95 U/L). Total protein was within reference interval (57 g/L; 54-75 g/L). The dog was treated with one dose of enrofloxacin (dose unknown) and sent home with the instructions to recheck the following day. Repeat CBC at the regular veterinary clinic 24 hours later showed a precipitous decline in hematocrit (8%), with worsening of vomiting and lethargy, and the dog was referred to OVCTH. Results of general physical examination at OVCTH revealed severe tachycardia (HR = 200 bpm), a grade I/VI systolic heart murmur, and pale white mucous membranes. CBC confirmed the presence of a severe,
macrocytic and hypochromic anemia (HCT = 8%; MCV = 76 fL; MCHC = 279 g/L) that was accompanied by microscopic autoagglutination and spherocytosis. Absolute reticulocyte count (364.1 x 10⁹/L) demonstrated a strong regenerative response; thus, rubricytosis (9.9 x 10⁹/L) was deemed appropriate. The systolic heart murmur found at physical exam was attributed to the severe anemia.

Serum chemistry showed the following abnormalities: mild hypokalemia (3.2 mmol/L; 3.8-5.4 mmol/L) referable to gastrointestinal loss and reduced intake; mildly increased urea (11.9 mmol/L; 3.5-9.0 mmol/L) with normal creatinine, likely due to GI hemorrhage or early reduction in GFR due to dehydration; marked hyperbilirubinemia (total bilirubin 82 umol/L; 0-4 umol/L) composed of elevations in both free and conjugated forms, which was attributed to severe, acute hemolysis and cholestasis; increased alkaline phosphatase activity (levamisole sensitive, 235 U/L; 22-143 U/L) due to cholestasis; and increased ALT activity (277 U/L; 19-107 U/L) referable to hepatocellular damage, most likely a result of hypoxic injury. Serology for heartworm antigen and for tick-borne diseases was negative, and results of abdominal ultrasonography were unremarkable.

Immunosuppressive therapy was commenced, and after 2 days and one packed RBC transfusion, the dog’s PCV was stable at 21%. However, she continued to vomit, and a course of metoclopramide (1 mg/kg/day constant rate infusion) and ondansetron (0.1 mg/kg IV q6h) was started. Endoscopy revealed a few small pyloric erosions and Helicobacter sp. was cultured from the stomach; however, the clinical significance of the bacterial culture result was undetermined, and the vomiting resolved within a few days after anti-emetic therapy. The dog was discharged after 7 days in hospital. She continued
to improve over the ensuing 4 months, and at her last follow-up visit at OVCTH, PCV was 51%.

Case 11 was a male castrated Cocker Spaniel who was 10 years old at his initial visit. He presented to his regular veterinarian for acute onset lethargy and anorexia of several days duration, and orange colored urine. The dog had previously been healthy, was not on medications at the time, was current on vaccines, and had not travelled outside Ontario. The regular veterinarian assessed the dog to be ill enough to require intensive medical care, and sent the dog immediately to OVCTH. On initial presentation, the dog had normal vital signs but very pale icteric mucous membranes. There was moderate hepatosplenomegaly, mild bilateral lenticular sclerosis, and mild otitis externa of both ears. CBC at OVCTH revealed marked macrocytic, hypochromic and markedly regenerative anemia (HCT = 16%; MCV = 91 fL; MCHC = 284 g/L; absolute reticulocyte count = 463.1 x 10^9/L) accompanied by autoagglutination and spherocytosis. Serum chemistry showed mild hyperbilirubinemia (total bilirubin 9 umol/L; conjugated bilirubin 3 umol/L, reference interval 0-1 umol/L; and free bilirubin 6 umol/L, reference interval 0-3 umol/L) and increased alkaline phosphatase activity (719 U/L, reference interval 22-143 U/L), which were attributed to a combination of hemolysis and cholestasis. A complete work-up failed to reveal an underlying cause for the hemolysis. The dog was initially treated with a course of immunosuppressive drugs (dexamethasone and azathioprine), gastrointestinal protectants (famotidine), aspirin and intravenous fluids (Plasmalyte A) at dosages previously described. However, after a few doses of azathioprine, he began to vomit; azathioprine was discontinued and a course of cyclosporine (5 mg/kg PO q12h) was commenced. He gradually improved and was discharged 4 days later after his PCV and clinical condition had stabilized. He was doing well 4 months after discharge, with a
PCV of 44% (taken at his regular veterinary clinic), but within a few days of that follow-up visit, the dog developed profuse vomiting. Thorough work-up at his regular veterinarian revealed markedly elevated amylase, lipase and ALT activities. He was treated at his regular veterinary clinic for pancreatitis and indeterminate liver disease, but continued to deteriorate pending referral to OVCTH for further work-up and care. His owners elected humane euthanasia. A necropsy was not performed.

**Case 12** was a male castrated Whippet that was 9 years old at the time of initial presentation. His owners sought veterinary care when the dog had been lethargic, anorectic and had been vomiting what appeared to be paper towels. The dog was generally well-supervised and was not previously ill or currently on medications other than heartworm preventatives. On general physical examination by the regular veterinarian, the dog exhibited abdominal pain. Radiographs were suggestive of a gastrointestinal foreign body, but none was found during an exploratory laparotomy. Results of in-house blood work taken prior to surgery or treatment showed a mild anemia (HCT = 31%), but the veterinarian also noted the presence of agglutination. The following day, repeat in-house blood work showed worsening anemia (29%) and marked hyperbilirubinemia (total bilirubin 50 μmol/L; 0-5 μmol/L). The veterinarian administered 200 mg IV of prednisolone sodium succinate (Solu-Delta-Cortef, Pfizer, NY, USA) and 500 mg sucralfate PO, and referred the dog to OVCTH.

General physical exam at OVCTH was unremarkable except for the presence of pale mucous membrane, a grade III/VI systolic heart murmur, and laparotomy incision. CBC confirmed the presence of a moderate to marked macrocytic anemia (HCT = 18%; 39-56% and MCV = 85 fL; 62-72 fL) accompanied by marked agglutination, spherocytosis, and presence of occasional ghosts. Total protein was within reference
intervals. The anemia was non-regenerative at the time of admission (absolute reticulocyte count = 18.0 x 10^9/L); however, thorough work-up, including serology for tick-borne diseases and heartworm, did not disclose an underlying cause for the immune-mediated hemolysis. The lack of a robust regenerative response at that time was attributed to acute onset of the disease (i.e. before regenerative response could be seen) or possibly the presence of immune-mediated disease involving erythrocyte precursors, i.e. pure red cell aplasia (Weiss, 2002). Additionally, CBC showed a mild leukocytosis (24.5 x 10^9/L) composed of mild neutrophilia with left shift, and monocytosis (2.21 x 10^9/L), consistent with inflammation. Serum chemistry abnormalities included moderate to marked hyperbilirubinemia (34 umol/L), mild hypercholesterolemia (11.20 mmol/L; 3.60-10.20 mmol/L), and mildly elevated alkaline phosphatase activity (318 U/L), which were referable to a combination of cholestasis and hemolysis.

The dog was treated with immunosuppressive drugs (dexamethasone and azathioprine), famotidine, and intravenous fluids, at previously described dosages. On day 2, his HCT declined to 13%, but this was accompanied by >10-20 polychromatophils per 100x objective, indicating continued hemolysis and appearance of regenerative response. He was given a packed RBC transfusion, and after four days deemed stable enough to be transferred back to his regular veterinarian for continued care (at discharge, his PCV was 25% and total solids were 77 g/L). The dog’s hematocrit at a follow-up visit with the regular veterinarian two months after discharge was 40%, and he appeared to be clinically improved.

**Case 13** was a male castrated Golden Retriever that was 6 years of age at the time of presentation. The dog presented to his regular veterinarian with a 2 day history of lethargy and anorexia. General physical examination abnormalities included icterus,
tachycardia and weak femoral pulses. In-house blood work showed severe anemia (PCV = 9%) and hyperbilirubinemia. A blood smear prepared at the regular veterinarian’s clinic showed increased polychromasia. The dog was deemed unstable, and immediately transferred to a local emergency veterinary clinic for overnight care. Treatments included IV fluids (rate and type unknown) and one dose of IV dexamethasone (dose unknown). The following morning, the dog was sent to OVCTH. Abnormalities on initial CBC at OVCTH included severe, macrocytic and regenerative anemia accompanied by spherocytosis, RBC ghosts and agglutination (HCT = 9%; MCV = 76 fL; absolute reticulocytes 136.9 x 10^9/L); moderate leukocytosis (42.4 x 10^9/L) composed of mature neutrophilia (36.04 x 10^9/L) and monomacytosis (2.97 x 10^9/L); and an appropriate rubricytosis (2.54 x 10^9/L). Abnormalities on serum chemistry profile included increased urea (13.6 mmol/L; 3.5-9.0 mmol/L) with normal creatinine (attributed to early decrease in GFR due to dehydration or GI hemorrhage); marked hyperbilirubinemia (87 umol/L; 0-4 umol/L) composed of equal magnitude elevations of conjugated and free bilirubin, and mildly elevated alkaline phosphatase activity (227 U/L) which were referable to hemolysis and cholestasis; moderately increased alanine aminotransferase (ALT) activity (541 U/L) indicative of hepatocellular damage, most likely due to hypoxic injury; and mild to moderate increases in creatine kinase (3182 U/L; 40-255 U/L), amylase (2540 U/L; 299-947 U/L), and lipase (2784 U/L; 60-848 U/L) activities. The latter changes were indicative of skeletal muscle damage (likely hypoxic injury and/or intramuscular injections) and pancreatic injury (e.g. pancreatitis or pancreatic hypoxia), respectively, or prolonged half-life of enzymes due to decreased GFR. Immunosuppressant therapy (dexamethasone and azathioprine at previously described dosages), low-dose aspirin and IV fluids (Plasmalyte A) were administered, as was 1 unit of packed RBCs. During the
first night at OVCTH, the dog developed ventricular premature contractions, and lidocaine (100 ug/kg/min constant rate infusion) was given. Echocardiograms showed mild aortic stenosis and the presence of a fibrin tag on the aortic valve, thus a course of sotalol (2 mg/kg PO q12h) was started due to the risk of thromboembolism. Over the course of the next 3 days, the dog’s PCV continued to decline despite immunosuppressive therapy and repeated packed RBC transfusions (a total of 3 were given). Cyclosporine (5 mg/kg PO q12h) was added to the immunosuppressant regimen, but due to poor prognosis and continued clinical deterioration, the dog was euthanized on the 4th day of hospitalization.

**Case 14** was a 3 year-old male castrated Cocker Spaniel who was presented to a local emergency clinic for progressive lethargy and anorexia of a few days’ duration, and acute onset “red colored urine.” On arrival to the emergency clinic, the dog also had one episode of vomiting. The dog was well-supervised, with no history of previous illness or current medications other than heartworm preventatives. There was no history of dietary indiscretion or toxin exposure, or travel outside Southern Ontario. In-house lab work revealed a severe anemia of 13% associated with microscopic agglutination. Leukogram consisted of mild mature neutrophilia (17.4 x 10^9/L; 2-12 x 10^9/L) and mild monocytosis (2.34 x 10^9/L; 0.3 – 2.0 x 10^9/L), which were consistent with inflammation. The attending veterinarian at the emergency clinic started intravenous fluids (rate and type unknown) and administered 1 dose of intravenous dexamethasone (dosage unknown). The following day, the dog was transferred back to his regular veterinarian, who deemed the dog clinically unstable, and thus sent the dog directly to OVCTH for continued care. Abnormalities on general physical examination included white mucous membranes and tachycardia (HR = 200 bpm). CBC at OVCTH showed a precipitous worsening of anemia
(HCT = 6%), which was associated with ghost cells, autoagglutination and occasional spherocytes. Additionally, the anemia was macrocytic (MCV = 79 fL) and regenerative (absolute reticulocyte count = 100.8 x 10^9/L). Although a component of rubricytosis (22.65 x 10^9/L) could be attributed to regenerative response, it was considered too high given the degree of reticulocytosis, and therefore inappropriate. This was thought to be a combination of hypoxic injury to bone marrow, glucocorticoid administration and possibly reduced splenic function (Stockham and Scott, 2008). The dog also had a marked leukocytosis (corrected WBC = 60.5 x 10^9/L) consisting of moderate to marked neutrophilia (43 x 10^9/L) with left shift (bands = 3.91 x 10^9/L) and monocytosis (3.91 x 10^9/L), which were indicative of inflammation. Serum chemistry abnormalities included hyperbilirubinemia (total bilirubin = 10 umol/L; 0-4 umol/L) consisting primarily of increased free bilirubin (9 umol/L; 0-3 umol/L), which was referable to acute hemolysis. The red color of urine was attributed to intravascular hemolysis and consequent hemoglobinuria. IV fluid therapy, immunosuppressive drugs (including dexamethasone, azathioprine, and cyclosporine at dosages previously described), and a total of 2 packed RBC transfusions were administered. Over the next several days, the dog continued to improve clinically, and was discharged on day 6 to the care of his regular veterinarian. The regular veterinarian contacted OVCTH several times over the course of the following 8 months to report that the dog had been doing well (PCV stable at 38%) and had been weaned off his prednisone and cyclosporine, but was continuing azathioprine at a much reduced dose.

**Case 15** was a 10-year-old female spayed mixed breed dog who was presented to her regular veterinarian for acute onset lethargy, anorexia and excessive panting of about 5 days duration. The dog had previously been healthy and was not on medications other
than heartworm preventatives. There was no history of travel outside of Ontario. The physical examination did not reveal any significant abnormalities other than pale mucous membranes. An in-house CBC revealed anemia (HCT = 19%, 37-55%). Because the dog appeared to be clinically deteriorating, he was referred to OVCTH that same evening. On admission, CBC revealed rapidly worsening anemia (HCT = 13%) with marked autoagglutination and spherocytosis. The anemia was regenerative (absolute reticulocyte count = 125.6 x 10^9/L). Additionally, mild leukocytosis (15.8 x 10^9/L) with neutrophilia, left shift and monocytosis – consistent with inflammation – were also noted. Serum chemistry profile was unremarkable except for mild increase in amylase activity (1174 U/L; 299-947 U/L). Serology for heartworm and tick-borne diseases was negative, and total body imaging was unremarkable. Treatments included immunosuppressive drugs (dexamethasone and azathioprine) and aspirin at previously described dosages, as well as intravenous fluids. Over the course of 72 hours, the dog improved clinically and PCV stabilized to 22%. She was subsequently discharged to her regular veterinarian for continued care. She continued to improve clinically, and eight months after discharge, her HCT was within reference interval (43%). The case was lost to follow-up after this time.

**Non-IMHA anemic dogs**

A total of four (4) dogs were selected for this group. They ranged in age from 3 to 12 years (mean ± SD, 7.3 ± 4.4 years) and included one each of Weimaraner, Shih Tzu, Golden Retriever, and Saint Bernard. Hematocrit at the time of admission ranged from 18% to 31% (mean ± SD, 24.5 ± 6.5%) (see Table 2).
Table 2: Clinical and laboratory findings of dogs with non-immunologic anemia.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yrs)</th>
<th>Signalment</th>
<th>Hct</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12</td>
<td>FS\textsuperscript{c} Shih Tzu</td>
<td>0.34</td>
<td>Iron deficiency, chronic external blood loss due to transitional cell carcinoma of the urinary bladder</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>MC\textsuperscript{d} Golden retriever</td>
<td>0.31</td>
<td>Mast cell tumour</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>MC St. Bernard</td>
<td>0.29</td>
<td>Chronic renal insufficiency</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>MC Weimeraner</td>
<td>0.18</td>
<td>Splenic torsion</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean ± SD = 7.3 ± 4.4
\textsuperscript{b}Hct = hematocrit, mean ± SD = 0245 ± 0.65
\textsuperscript{c}FS = female spayed
\textsuperscript{d}MC = male castrated
Case A was a 12-year-old female spayed Shih Tzu that was initially presented to OVCTH for further evaluation of unexplained anemia. Relevant medical history provided by the regular veterinarian included: 3 year history of keratoconjunctivitis sicca, which was stable and managed with ophthalmic tacrolimus; one episode of pyometra 2 years previous, which was managed surgically; and chronic, intermittent axillary dermatitis (yeast infection), for which the dog received chlorhexidine baths as needed. The anemia (HCT = 31%; 39-60%) was an incidental finding by her regular veterinarian, during routine pre-anesthetic blood work for dental prophylaxis. However, a follow-up CBC one month later confirmed that the anemia was persistent (HCT = 20%; 30-56%), microcytic (MCV = 53 fL; 62-72 fL), hypochromic (MCHC = 327 g/L; 330-360 fL), and poorly regenerative (absolute reticulocyte count 60.4 x 10^9/L) in nature. The most likely explanation given at the time was iron deficiency anemia secondary to chronic external blood loss, but a source of hemorrhage could not be found despite an exhaustive search. No specific treatments were recommended for the anemia at the time. However, four months later she presented again to OVCTH for a 1-2 month history of waxing and waning hematuria that was not responding antibiotic therapy. Blood from Case A was collecting during this visit. Abnormalities on CBC included mild anemia (HCT = 34%; 39-56%), microcytosis (MCV = 55 fL; 62-72 fL); hypochromasia (MCHC = 312 g/L; 330-360 g/L); and mild, mature neutrophilia without toxic changes (17.4 x 10^9/L; 2.9-10.6 x 10^9/L). Serum chemistry was unremarkable. The regular veterinarian had sent a urine sample to a reference laboratory two days prior. Chemical analysis of the urine was unremarkable, other than the presence of blood and protein. The clinical pathologist reported large numbers of RBCs (10-20 per high power field), WBCs (> 3-5), and many rafts of epithelial cells which displayed “dysplastic changes.” However, the significance
of these cells was not known given the presence of blood and inflammation. A cause of hematuria and dysplastic epithelium (e.g. cystic calculi) was not found during abdominal ultrasonography, and bacterial culture was unrewarding. Over the course of several months, episodes of hematuria progressed to stranguria, and further work-up at OVCTH revealed intravesicular as well as metastatic transitional cell carcinoma. Palliative chemotherapy with piroxicam was commenced, and the dog was still alive at the time of manuscript preparation.

**Case B** was a 10-year-old, male Golden Retriever that presented to OVCTH for evaluation of a mast cell tumor over the right stifle. Five months prior to referral, the regular veterinarian had removed a mass from the right stifle, and histopathology revealed that it was a grade 2 mast cell tumor. However, a larger mass re-appeared at the original surgical site and fine needle aspirate cytology confirmed it was a mast cell tumor. Blood samples were taken on admission to OVCTH, and the only abnormality noted was a mild, normocytic, normochromic, non-regenerative anemia (HCT = 31%; 39-56%) which was attributed to anemia of chronic disease (neoplasia). The dog had received an anti-inflammatory dose of prednisone PO and 25 mg diphenhydramine IM prior to referral, to reduce edema and swelling of the leg. The mass was excised, and the dog received chemotherapy (vinblastine 0.2 mg/m² IV every week) over the course of four weeks. However, due to declining clinical condition and poor prognosis, the owners elected euthanasia at their regular veterinary clinic. A necropsy was not performed.

**Case C** was a 3-year-old female St. Bernard who was referred to OVCTH for evaluation of persistent anemia. The anemia was first noted by her regular veterinarian about six months prior during pre-anesthetic blood work, for surgical management of pyometra. In-house blood work showed a marked inflammatory leukogram, to be
expected with pyometra, as well as mild, normocytic and normochromic anemia, which was attributed to inflammatory disease. The chemistry profile showed moderate azotemia. The dog recovered uneventfully, but over the course of several follow-up visits, the anemia and azotemia persisted. In addition, the owners also noted polyuria. Abnormalities on initial CBC, chemistry profile, and urinalysis at OVCTH included the following: HCT = 29% (39-55%); reticulocyte count = 45.3 x 10⁹/L; urea = 18.4 mmol/L (3.5-9.0 mmol/L); creatinine = 444 umol/L (20-150 umol/L); urine specific gravity = 1.012 with 0.2 g/L SSA protein. These changes were consistent with renal insufficiency and anemia of renal disease. Abdominal ultrasonography disclosed the presence of bilateral degenerative renal changes, which confirmed the suspicion of primary renal disease. Biopsy of the kidneys was recommended, but the owners declined further work-up and the case was lost to follow-up.

**Case D** (previously included as IMHA case 6) was a male castrated Weimaraner who was 4 years of age upon initial presentation to his regular veterinarian. The dog had a history of acute onset lethargy and diarrhea of 2-3 days duration. The dog also had several episodes of vomiting in the last 2 weeks. Initial blood work by the veterinarian disclosed mild to moderate anemia of 26% (37-55%) and normal total protein, 59 g/L (55-74 g/L). Additionally, there was a moderate leukocytosis (43.7 x 10⁹/L; 4.9-15.4 x 10⁹/L) composed of moderate neutrophilia (33 x 10⁹/L; 5.8-8.5 x 10⁹/L) and marked monocytosis (8.4 x 10⁹/L; 0-1.1 x 10⁹/L), which were changes consistent with inflammation. Over the course of 24 hours, the hematocrit continued to decrease, to 23%, while total protein remained constant. The dog was then referred to OVCTH with suspected primary IMHA. Hematocrit on arrival at OVCTH was 18%. However, examination of blood film failed to reveal any agglutination or spherocytosis, and absolute reticulocyte count (66 x 10⁹/L)
was consistent with poorly-regenerative anemia; these findings were not consistent with primary IMHA. Results of an abdominal ultrasound indicated the presence of splenic torsion, which was confirmed on exploratory laparotomy. A splenectomy was performed, and the dog recovered uneventfully, to be discharged from hospital 4 days later. The anemia in this case was attributed to a combination of inflammatory disease and acute internal blood loss/sequestration due to splenic torsion. Samples for this study were collected at the time of admission to OVCTH, but when it was learned subsequently that the dog did not have primary IMHA, the case was later included as non-IMHA, anemic patient group.

Control dogs

Dogs from this cohort ranged in age from 1 year to 14 years (mean ± SD, 5.8 ± 3.2 years) and were of various breeds, including 7 mixed breed dogs; two each of Labrador retriever, Golden retriever, and Belgian Tervuren; and one each of standard poodle, Staffordshire bull terrier, Greyhound, and German shepherd. All dogs were deemed healthy based on physical examination and history, and had negative serology for heartworm antigen. None of the dogs was on medications other than heartworm preventative, or had a history of previous blood transfusion or (if female) pregnancy. CBC and serum chemistry profiles of all dogs were unremarkable.

1D SDS-PAGE, erythrocyte lysates (ghosts)

Pooled RBC membranes (lysates or RBC ghosts) prepared from control dogs were analyzed by 1D SDS-PAGE and stained with silver to show the major integral protein components. The results are presented in Figure 1 and are consistent with previously published work (Barker, 1991; Steck, 1974). Five bands, labeled according the accepted
nomenclature (Steck, 1974) are clearly visible, although fainter bands of molecular masses smaller than 37 kDa can also be seen. Since the intensity of the band on the gel is proportional to the relative quantity of protein as well as the amount of time the gel is incubated in developer, it is not unexpected that band 3 (anion exchanger 1) is the thickest and darkest of bands, being the most abundant of RBC membrane proteins (Bruce et al., 2003). Faintly visible bands, representing lower-abundance proteins (bands 6, 7 and 8 according the nomenclature by Steck, 1974), were visualized by leaving the gel in developer for a longer period of time, but this caused overdevelopment and thus poor resolution of other protein bands (not shown).

Validation of xylene elution technique

In order to validate the xylene elution technique, DEA 1.1-positive RBCs from blood donor dogs were sensitized with polyclonal antiserum specific for DEA 1.1. Eluates from sensitized RBCs (“positive control eluates”) were then analyzed by 1D SDS-PAGE and immunoblotting to confirm the presence of immunoglobulin. Figure 2 shows the immunoblot of positive control eluate (lane 1) and of purified canine IgG (lane 2, VMRD Inc., Pullman, WA, 1:20 dilution), reacted against polyclonal sheep anti-canine IgG conjugated to HRP. If the sample contained IgG, the expected pattern of reactivity would include 2 bands: one at ~50 kDa (corresponding to Ig gamma heavy chain), and one at ~20 kDa (corresponding to Ig light chain). As expected, reactivity at a 50 kDa band was seen in lane 2 (purified canine IgG), and also lane 1, which indicated the presence of Ig gamma heavy chain in positive control xylene eluate. Moreover, there was reactivity at ~20 kDa in purified canine IgG (Fig. 2, red circle), and in positive control xylene eluate, although the band was wider and much more prominent in the latter sample. Since all
xylene eluates were heavily stained with hemoglobin, it was very likely that in addition to light chain, this ~20 kDa band also included hemoglobin, which in some species is known to non-specifically bind antibodies used in immunoblots (BA Quinn, unpublished observations).

Several unexpected bands of reactivity were also seen in the positive control eluate. Two bands migrating between 40 and 50 kDa were present in both lanes. It is possible that these bands represented different IgG subclasses or IgG with alternative glycosylation. Additionally, there was reactivity at a ~37 kDa band in both positive control eluate and purified canine IgG, which was surprising because no components of IgG are known to migrate at this apparent molecular mass. The secondary antibody used clearly recognizes antigens other than IgG. Although the manufacturer employed immunoelectrophoresis to confirm that the sheep antibody only recognizes canine IgG, and not other classes (e.g. IgM, IgA or IgE), immunoelectrophoresis does not rule out the possibility that other components of canine serum were recognized by the sheep antibody. The manufacturer stated that the antigen source for immunization of sheep was “highly purified,” but not necessarily affinity purified, thus the sheep could have been inadvertently immunized against other contaminatory components of canine serum, such as complement C3 fragments. More importantly, activation of serum complement (which can happen spontaneously) can result in the binding of C3d (which has a molecular weight of ~35 kDa) to IgG (Lachmann et al, 1982). Consequently, C3d would likely be present in “purified IgG” preparations (including the one used to immunize the sheep and the “purified canine IgG” sourced from VMRD Inc.). In addition, C3d is known to accrue on red cells of various species as part of the red cell ageing (Shapiro et al, 1994), and thus its presence in eluates would also not be unexpected. Thus, it is possible that reactivity at
the ~37 kDa band could be against complement fragment C3d. However, it is impossible to definitively ascertain the identity of this or any of the additional “unexpected” bands without further analysis (e.g. mass spectrometry).

**Blue native PAGE of DEA 1.1-positive RBC lysates**

Pooled RBC lysates were analyzed by blue native PAGE. The purpose of this experiment was to characterize RBC membrane proteins in non-denaturing conditions, and to validate the use of immunoglobulin from xylene eluates as a primary antibody in an immunoblot application. Non-denaturing PAGE was chosen because results of previous work by Corato and colleagues (1997) indicated that the epitopes recognized by polyclonal antibodies against DEA 1.1 (produced by alloimmunization) are likely conformational. Regular non-denaturing or native PAGE was deemed unsuitable for separation of membrane integral proteins: native PAGE takes place in an ionic or aqueous environment, and solubilization of membrane, even with relatively gentle detergents such as Triton X-100, causes aggregation of membrane integral proteins, due to the hydrophobic nature of the transmembrane domain. The problem of aggregation is dealt with in two ways in BN PAGE. First, the method uses amphipathic detergents such as digitonin, which form thermodynamically stable micelles with hydrophobic cores. These micelles provide a suitable environment for the dissolution of hydrophobic domains of proteins without denaturation (Reisinger & Eichacker, 2006; Wittig et al, 2006). Second, Coomassie blue G250 stain further reduces protein aggregation formation by binding to the hydrophobic domains of proteins, and it also induces a negative charge shift, which allows the proteins to migrate toward the anode (even at running pH of 7.5) (Schagger et al., 1994).
Figure 3 shows the results of BN PAGE analysis of DEA 1.1-positive RBCs. Although proteins transferred successfully to nitrocellulose (based on positive Ponceau S staining), no reactivity was obtained with polyclonal antiserum specific for DEA 1.1, or with xylene eluates from sensitized red cells (blots not shown).

**Immunoblot experiments**

**Experiment 1: Pooled canine RBC lysate as target**

Eluates from 11 IMHA patients, 4 non-IMHA anemic dogs, and 2 healthy control dogs (both blood donors) showed specificity against two proteins, one at ~100 kDa and another at ~50 kDa (Fig. 4). Based on results of LC-MS/MS and Scaffold analysis, the former (~100 kDa) was band 3 (anion exchanger 1, AE1), while the latter (~50 kDa) was IgG heavy chain. In humans and dogs, it has been shown that recognition of a neoantigen related to band 3 (so-called senescent cell antigen, which likely represents oxidant-damaged band 3 and/or hemichrome-induced clustering of band 3) by autologous IgG represents an important mechanism for red cell turnover: Accumulation of specific IgG above a relatively low threshold (about 200 IgG molecules per erythrocyte) triggers binding and phagocytosis of senescent erythrocytes by macrophages of the mononuclear phagocyte system (Kay, 1984; Rettig et al, 1999; Arese et al 2005; Bosman et al, 2005). In addition, seldom is any elution technique 100% efficient (South et al, 1986). Therefore, it is very likely that some immunoglobulin remained adherent to membranes during lysate preparation, explaining its presence in PAGE analysis of RBC lysates, and hence immunoblot reactivity with sheep anti-canine IgG. There was insufficient eluate for this experiment from cases 5, 11 and 15.
Eluates from 4/11 (36%) IMHA cases (cases 1, 4, 9, and 12), 2/4 (50%) non-IMHA patients (cases B and D), and 0/2 healthy blood donor dogs showed reactivity against a ~22 kDa protein, whose identity was peroxiredoxin 2 (Prx 2), based on LC-MS/MS analysis. Prx 2 is a member of the 2-cysteine peroxidase family that has recently been shown to play a non-redundant role in anti-oxidant defense in the erythrocyte, alongside better-studied systems such as glutathione peroxidase and catalase (Low et al, 2008). Typically, Prx 2 exists in the cytosol of the erythrocyte (Low et al, 2008), but has been linked to erythrocyte membranes in vivo and in vitro in severe oxidative stress (Low et al, 2007; Rocha et al, 2008; Rocha et al, 2009). Results may therefore reflect increased oxidative stress in a subset of IMHA and non-IMHA patients, although further studies would be required to confirm this finding (e.g. immunoblot using anti-Prx 2 antibodies, other markers of oxidative stress).

**Experiment 2: Eluate as target**

Since no elution technique is 100% efficient (South et al, 1986; Judd, 1993) and the particular technique used ultimately resulted in lysis of the red cell, there was a possibility that any antigen of interest was still associated with cognate antibody, and thus found in eluate. More importantly, bound antigen could potentially cause steric hindrance of antibody binding in the immunoblot experiment 1 (above), resulting in false negative. For this reason, we also performed an immunoblot experiment with patient eluate as antigen (RBC antigens contained in eluate) and autologous plasma as primary antibody (Fig. 6). Sufficient sample was available for all IMHA patients, except for case 14.

Table 2 summarizes the results of this experiment. Plasma of IMHA patients had on average reactivity against a greater number of bands than plasma of non-IMHA patients (Fig. 5). A total of 6 bands could be seen on immunoblots prepared from IMHA
patient eluates (numbered 1 to 6); serum from IMHA patients had on average reactivity against 4 bands; 7/12 IMHA patients had reactivity against at least 5 of these (4/12 had reactivity against all 6); and serum from healthy dogs had no visible bands of reactivity.

LC-MS/MS analysis of bands from 1D SDS-PAGE often yields more than one candidate protein, since more than one protein in a complex mixture may have similar molecular masses, and therefore migrate to a similar position in the gel. The potential list of proteins received from LC-MS/MS analysis was narrowed based on predicted molecular masses. Eleven of twelve (91.7%) IMHA patients had reactivity at a ~50 kDa band, while only 1 of 4 (25%) non-IMHA patients (only Case D, the dog with splenic torsion) and none (0/2) of the healthy dogs had reactivity at this band. LC-MS/MS identified immunoglobulin gamma heavy chain in this band, indicating that eluates from IMHA patients were more likely to contain immunoglobulin gamma (heavy chain) than those from non-IMHA patients and healthy control dogs.

Plasma from 4/12 IMHA patients, 0/4 non-IMHA anemic dogs, and 0/2 healthy dogs had reactivity against a ~170 kDa protein band, which was a portion of complement factor C3 based on LC-MS/MS. Again, this finding is consistent with the proposed mechanism of primary IMHA, where hemolysis is caused by the presence of erythrocyte-bound antibody and/or complement (Warman et al, 2008).

Many of the proteins identified in eluates of IMHA patients by LC-MS/MS were cytosolic, and their presence was probably due to unavoidable contamination by cytoplasmic matrix contents, including hemoglobin, which was evident in many of the gels as a thick band at ~16 kDa. These proteins were not considered likely candidate autoantigens due to their cytosolic location (Table 3).
Clinical characteristics of IMHA cases included in this study corroborate data from previous studies, which indicate that primary IMHA is a disease that tends to affect middle-aged, female dogs (Carr et al., 2002; Weinkle, 2005; Elwood & Polton, 2008; Piek et al, 2008). In addition, the results of this study further support the proposed pathogenic mechanism; that is, the coating of erythrocytes with immunoglobulin and/or complement leads to premature removal of RBC from circulation by macrophages in the spleen and the liver (Al-Ghaszlat, 2009; Morely et al, 2008), which results in severe anemia. RBC eluates from IMHA patients detected a greater number of red cell membrane antigens than those of non-IMHA and healthy dogs. Moreover, the bands corresponding to immunoglobulin heavy chain (around 50 kDa) in IMHA patients appeared semi-quantitatively more intense than those from non-IMHA anemic dogs and healthy dogs. These findings were consistent with those of Morley and others (2008), who found that anemic dogs, particularly those with IMHA, had a significantly higher percentage of Ig-positive RBCs than non-anemic dogs by flow cytometric analysis. Although the concentration of Ig in eluates was not quantified in this study, samples were processed in an identical fashion. Hence, it is unlikely that differences between patient and control dogs were due to differences in volume of eluate obtained or analyzed from each patient, but rather that they truly reflect features of the disease. None of the healthy control dogs and only one of the non-IMHA anemic dogs had evidence of Ig in red cell eluates. As mentioned previously, low levels of autoreactive, specific IgG would also be expected on senescent red cells of healthy individuals (Kay, 1984; Arese et al, 2005; Bosman et al, 2005), but perhaps the level of Ig coating senescent erythrocytes of healthy dogs is too low for efficient elution, and too low therefore to be detected by immunoblot. Dumaswala
et al (1994) determined the IgG concentration of RBC eluates from sensitized RBCs and from individuals with warm (IgG-mediated) AIHA to be far higher than that of eluates from non-sensitized RBCs, but even then the total IgG comprised <0.13% of total protein content.

Interestingly, Ig was identified in red cell eluate of one of the non-IMHA patients (case D, the dog with splenic torsion). The significance of this finding is not known, although Morley et al (2008) demonstrated that 8.3% of non-anemic dogs with a variety of diseases had Ig-positive RBCs (above a threshold set for what is expected in healthy dogs) based on flow cytometry. It is possible that in this patient there were greater numbers of senescent red cells in circulation due to increased splenic sequestration and decreased splenic removal, and therefore greater number of exposed “senescent cell antigen” available for binding by immunoglobulin.

Humans, mice, and rats have different subclasses of IgG (IgG1, IgG2, IgG3 and IgG4 in humans; IgG1, IgG2a, IgG2b, and IgG3 in mice; and IgG1, IgG2a, IgG2b, and IgG2c in rats), which display different biological properties (Kindt et al, 2007). For example, complement activation via the classical pathway is initiated most effectively by IgG3 in humans, and IgG2a and IgG2b in mice (Kindt et al, 2007). In addition, the activity of IgG subclasses in human autoimmune diseases have been extensively studied (e.g. IgG2 subclass predominating in Hashimoto’s thyroiditis; IgG1 subclass predominating in type 1 diabetes) (Day, 1996).

Dogs also have four IgG subclasses, corresponding to four different Ig gamma heavy chains (Tang et al, 2001). There is, however, some confusion regarding nomenclature (Day, 2007). Reynolds and Johnson (1970) were first to name the subclasses IgGA, IgGB, IgGC, and IgGD, and it is this nomenclature that Tang and
colleagues (2001) used when they cloned and sequenced cDNAs of the four different canine Ig gamma chains (see Table 3, GenBank accessions are entered as immunoglobulin gamma chains A through D, and subclasses B and C were identified in patient eluates). In an effort to be more consistent with nomenclature used in other species, the group at the University of Bristol (see Day, 2007) renamed the four different subclasses IgG1 to IgG4. The criteria for renaming were the relative serum concentrations (IgG1 > IgG2 > IgG3 > IgG4) and relative electrophoretic mobility, with IgG1 and 3 showing a cathodal migration, and IgG2 and 4 show an anodal migration (Day, 2007). Day (1996) demonstrated that IgG1 and 4 were the predominant subclasses involved in canine IMHA, but how the subclasses under this newer nomenclature system correspond to those of the previous system (A through D) is not known, since different panels of antibodies were used to establish each system. More importantly, formal studies of the function of each subclass in dogs – in terms of their relative ability to fix complement, opsonize, participate in antibody dependent cell-mediated cytotoxicity, and bind different types of antigens – are lacking (Day, 2007), and therefore conclusions regarding pathologic significance of subclass predominance in IMHA cannot be made.

In contrast to previous work (Baker et al, 1991; Barker & Elson, 1995), we did not find glycophorins on our list of potential autoantigens in any of our IMHA patients. Glycophorins are the most abundant group of red cell membrane glycoproteins (Chasis & Mohandas, 1992; Sato et al, 2008). In humans, five are known: glycophorins A, B and E are the products of three related genes, while glycophorins C and D arise from a single gene by alternative mRNA splicing (Chasis & Mohandas, 1992). These proteins are heavily glycosylated, and the large amounts of sialic acid they bear contribute roughly 60% of the overall negative charge of the red cell surface, and prevent RBC aggregation
Differences in results between this study and the one by Barker and Elson (1995) may be due to differences in techniques used to investigate candidate RBC autoantigens: the investigators of the previous study immunoprecipitated red cell antigens with patient eluates, while in our study we chose to use denaturing SDS PAGE-based immunoblots. It is possible that autoantibodies recognize a conformational epitope (or possibly carbohydrate moiety), which was altered during SDS PAGE, and therefore was not recognized by antibodies in patient eluates in experiment 1. Additionally, it is possible that heat treatment during eluate preparation could have denatured a conformational epitope that was co-eluted with antibody, resulting in lack of immunoreactivity in experiment 2. After the considerable technical challenges we faced in our attempts to optimize non-denaturing PAGE (and subsequent immunoblots) of RBC membrane proteins, we did consider performing immunoprecipitation as an alternative approach to both experiments, but were limited by the very small volumes of patient eluates available (often less than 300 uL per patient), time, and financial constraints.

In neither experiment did we probe for IgM reactivity (only sheep anti-canine IgG was used) which could have resulted in the omission of candidate autoantigen recognized only by autoreactive IgM. However, previous studies involving flow cytometry and monovalent Coombs’ reactivity demonstrated that IgG and C3 were far more commonly found to coat RBCs of dogs with IMHA than IgM (Day, 1996; Morley et al, 2008; Warman et al, 2008).

The results of this study suggest that peroxiredoxin (Prx) 2 was associated with RBC membrane in a subset of IMHA patients. To the author’s knowledge, this has not been previously reported. In mammals, there are six known isoforms of peroxiredoxin, which constitute a family of ubiquitous peroxidases that scavenge hydrogen peroxide
The major form in erythrocytes is Prx 2; at roughly 15 million copies per erythrocyte, it is the third most abundant cytosolic protein in the RBC (after hemoglobin and carbonic anhydrase), where it functions as part of the erythrocyte antioxidant system (Low et al, 2007; Low et al, 2008). In health, only ~0.05% of Prx 2 is bound to erythrocyte membrane, but increased binding of Prx 2 to the RBC membrane can be induced by oxidative stress in vivo and in vitro (Rocha et al, 2008; Low et al, 2008; Rocha et al, 2009). Rocha and colleagues (2009) hypothesized that Prx 2 simply accompanies oxidized hemoglobin to the membrane during hemichrome formation (since the two proteins are highly associated in the cytosol), while others have speculated that membrane linkage of Prx 2 serves a functional purpose, helping to reduce lipid hydroperoxides and thus protecting the membrane from oxidative damage (Low et al, 2008). Regardless of the role of Prx 2 membrane binding (which clearly requires further study), it serves as a marker for oxidative stress, and its association with RBC membrane in a subset of IMHA patients suggests that oxidative stress plays a role in the pathogenic mechanism of IMHA. Several non-IMHA patients (but none of the healthy dogs) also showed immunoreactivity to Prx 2, including one dog with a mast cell tumor and one with splenic torsion, indicating that the RBCs of these dogs were also faced with increased oxidative stress (possibly secondary to inflammation or chemotherapy, as in the case of the dog with mast cell tumor).

Superoxide dismutase 1 (SOD1) plays a central role in anti-oxidant defense of the red cell by scavenging superoxide, and not surprisingly SOD 1-deficient (SOD1−/−) mice have increased reactive oxygen species production compared to wild type mice and display an anemic phenotype, due to oxidant-mediated damage and increased turnover of RBCs (Iuchi et al, 2007). However, autoimmunity may also be an important cause of
anemia in the *SOD1*−/− mouse. Iuchi and colleagues (2007, 2009) demonstrated that lipid peroxidation of RBCs enhances autoimmunity through the production of 2 RBC membrane-associated neoantigens: 4-hydroxy-2-nonenal (HNE) and protein-bound acrolein. Not only were RBCs of *SOD1*−/− mice coated with greater quantities of Ig (as determined by flow cytometry) compared to wild type mice, but *SOD1*−/− mice had increased concentrations of circulating antibodies against acrolein and HNE. Furthermore, transgenic *SOD1*−/− mice that express human SOD1 (hSOD1) in an erythroid cell-specific manner had anti-HNE and anti-acrolein antibody concentrations that were no different than wild type, and were rescued from the anemic phenotype (Iuchi et al, 2007; Iuchi et al, 2009). These findings support the notion that in the *SOD1*−/− mouse, oxidative stress and lipid peroxidation were causes of autoantibody production. There is some evidence that oxidative stress is also involved in the pathogenesis of AIHA in NZB mice (see Iuchi et al, 2010): First, concentrations of lipid peroxidation products were higher in NZB mice compared to New Zealand White (NZW) mice, even at a young age (4 weeks), prior to onset of AIHA. Second, the levels of intracellular reactive oxygen species (ROS) increased significantly in NZB mice (but not in NZW mice) as they aged. Third, there was a significant positive correlation between severity of anemia and levels of intracellular ROS in NZB mice. And finally, transgenic expression of hSOD1 in NZB mice rescued the mice from AIHA phenotype, although evidence for specific defects in any of the known erythrocyte anti-oxidant mechanisms in the NZB mouse is still lacking (Iuchi et al, 2010).

Could oxidative stress be a causative factor in canine primary IMHA? Although the findings of the present study indicate that oxidative stress is likely involved in the pathomechanism of IMHA, the specific study design precludes the ability to make any further conclusions regarding oxidative stress acting as a trigger or cause for
autoimmunity, rather than being a result of autoimmunity. Clearly, this is an intriguing hypothesis that requires further investigation, particularly since there could be clinically relevant implications if proven correct (i.e. addition of anti-oxidant therapy to the treatment regime of IMHA patients). Although the specificities of antibodies were examined in the present study, the methods used did not allow for detection of antibody specificities against lipid peroxidation products such as HNE and acrolein. Thus, future efforts might include comparisons of anti-HNE and anti-acrolein antibody concentrations in IMHA vs. healthy dogs, specific measurements of oxidative stress indicators in IMHA vs. healthy dogs (e.g. thiobarbituric acid reactive substances), and analysis of genes for the anti-oxidative systems (catalase, SOD, glutathione peroxidase, etc.).

In a subset of IMHA patients (but in none of the control dogs) we also identified calpain 1 associated with RBC membrane. Members of the calpain family are ubiquitous calcium-sensitive cysteine proteases normally located within the cytosol of cells in an inactive form (Michetti et al, 1996). Increased cytosolic \([\text{Ca}^{2+}]\) is the main trigger for activation of calpain, which begins with autoproteolysis and translocation to the inner surface of the plasma membrane (Michetti et al, 1996). In nucleated cells, calpain plays a crucial role in apoptosis, where it activates pro-apoptotic factors such as Bax and Bid, and facilitates membrane blebbing by breaking down the cytoskeleton (Orrenius et al, 2003). There is growing evidence that erythrocytes also undergo an apoptosis-like process (eryptosis), which shares many morphologic features with apoptosis (e.g. cell shrinkage, membrane blebbing, externalization of membrane phosphatidylserine) (Bosman et al, 2005; Lang et al, 2005; Foller et al, 2008; Lang et al, 2008). Additionally, as in apoptosis, external stressors such as osmotic shock, oxidative stress and energy depletion can elicit eryptosis, and these stressors set in motion cell death via increased cytosolic \([\text{Ca}^{2+}]\) and
calpain activation (Foller et al., 2008). Recently, Head and colleagues (2005) demonstrated that binding of antibodies against glycoporphin C on human erythrocytes could trigger eryptosis. The results of this study suggest that IMHA patients have increased erythrocyte calpain activation, and thus apoptosis-like mechanisms (i.e. eryptosis) may play a role in the pathogenic mechanism of canine IMHA, possibly due to the increased presence of triggers such as oxidative stress (e.g. from inflammation) or due to ligation of autoreactive anti-RBC antibodies.

An unresolved issue is why there would be eluate and plasma immunoreactivity against calpain and Prx 2, respectively. It is unlikely that these proteins are dominant autoantigens in IMHA, since they are normally cytosolic except under conditions such as oxidative stress and eryptosis, which likely follow or accompany IMHA rather than trigger it. A major limitation of protein identification by tandem mass spectrometry from 1D SDS PAGE gels is that more than one protein may be present in each excised band; therefore, eluate or plasma immunoreactivity could have been against another protein of similar molecular mass. However, the remaining proteins identified were either of incorrect molecular mass for SDS PAGE gel migration pattern, or were exclusively cytosolic. An alternative explanation would be epitope spreading, i.e. diversification of epitope specificity from the initial, focused dominant epitope-specific immune response to other subdominant epitopes (Vanderlugt & Miller, 2002), although this would be impossible to prove.

The non-immunologic anemia cases in this study were chosen in an attempt to encompass as broad a range of etiologies of anemia as possible, so that any differences could be ascribed to the immunologic component of IMHA (as opposed to the anemia alone). However, we were limited logistically by the selection of cases that presented
concurrently with IMHA cases (so that samples could be processes as freshly as possible and under comparable conditions to the IMHA cases), and the availability of sample. Clearly, the study could have been improved and more robust conclusions could have been made had we included a greater number of cases in this cohort, and perhaps included a greater number of dogs with acute, regenerative anemias similar in severity to those of the IMHA dogs to better match the erythrocyte microenvironment. Nevertheless, the findings serve as an important starting point upon which further studies can expand.
CONCLUSIONS AND FUTURE DIRECTIONS

The objective of this study was to elucidate the dominant target antigens involved in canine primary IMHA by comparing the specificities of xylene eluates of RBC from dogs with IMHA to those of control dogs. Although the list of potential target autoantigens identified is limited, the results of this study support previously proposed mechanism of canine IMHA, and suggest that RBCs of IMHA patients are under oxidative stress. Studies in SOD1^{-/-} and NZB mice indicate that oxidative stress may enhance or predispose to autoimmunity through the production of autoantibodies against RBC lipid peroxidation products. Although the present study was limited in its ability to make such a conclusion in canine IMHA, this is an avenue of investigation that should be pursued since there may be important clinical implications. Further studies involving measurement of oxidative stress markers, analyses of genes involved in anti-oxidant defense of the RBC, and evaluation of antibody production against lipid peroxidation products are required. In addition, eryptosis, an apoptosis-like mechanism of erythrocytes, may also be involved in the pathogenic mechanism of IMHA, and may contribute to shortened RBC lifespan. Further studies directly probing eryptosis markers, e.g. direct measurements of calpain activation or annexin V binding, are needed to confirm the findings of the present study.

Limitations to identifying dominant target autoantigens may be related to technique. In particular, autoantibodies in canine IMHA may recognize conformational epitopes that are destroyed during denaturing SDS-PAGE analysis or during xylene elution. An alternative approach, such as immunoprecipitation of patient eluate with red cell membrane, may be warranted in future studies.
Figure 1: SDS-PAGE, silver stain, 15% acrylamide gel. Lane 1 = canine RBC membrane preparation; lane 2 = molecular weight marker ($M_R$)
Figure 2: Validation of xylene elution technique. Lane 1, undiluted xylene eluate of sensitized DEA 1.1-positive RBC; lane 2, 1:20 dilution of purified canine IgG (VMRD Inc., Pullman, WA, USA); arrow, 50 kDa; red circle, ~20 kDa (Ig light chain).
Figure 3: Blue native PAGE of purified porcine ficolin (lane 1) and DEA 1.1-positive canine RBC membrane preparation (lane 2), 4-15% gradient acrylamide, silver stain.
Figure 4: Immunoblots of pooled RBC membrane preparation as antigen, patient eluate as primary antibody, and polyclonal sheep anti-canine IgG conjugated to horseradish peroxidase as secondary antibody. Lane 1 = IMHA case 6; lane 2 = IMHA case 9; lane 3 = healthy blood donor dog, lane 4 = case A (iron deficiency anemia); lane 5 = IMHA case 1; lane 6 = IMHA case 9; lane 7 = IMHA case 7. Green circles indicate bands at ~100 kDa; yellow circles indicate bands at ~50 kDa; red circles indicate bands at ~22 kDa.
Table 3: RBC antigens in eluate reacted with autologous plasma from dogs with IMHA, other anemia or no anemia.

<table>
<thead>
<tr>
<th>Category</th>
<th>Case</th>
<th>RBC membrane protein&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
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<td>I&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>II&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> DEA1.1 positive
<br>
<sup>b</sup> DEA1.1 negative
<br>
<sup>c</sup> Approximate molecular weights: 20, 25-30, 50, 70, 100 and 170 kDa
Figure 5: Mean number of immunoreactive bands in each group of dogs (RBC eluate as antigen, autologous plasma as primary antibody, sheep anti-canine IgG as secondary antibody)
**Figure 6:** Immunoblots with RBC eluates as antigen, reacted against autologous plasma and sheep anti-canine IgG conjugated to horseradish peroxidase as secondary antibody. Lane 1 to 5 = IMHA cases 9, 12, 13, 4, and 11; lane 6 = case D (splenic torsion); lane 7 = healthy blood donor dog. Arrow, 50 kDa.
Figure 7: Left, immunoblot of IMHA case 7 (indicated by arrow with solid arrowhead, RBC eluate as antigen, autologous plasma as primary antibody), with band numbering system that corresponds to Table 3. Right, corresponding SDS-PAGE gel from which bands were excised for LC-MS/MS analysis. Lane 1, IMHA patient 7 showing bands 2 through 6 excised for LC-MS/MS analysis; lane 2, pooled RBC membrane preparation; lane 3, molecular weight marker; red arrow with open arrowhead = 50 kDa; red circles = band identified by LC-MS/MS as hemoglobin.
Table 4 (next page): Selected peptide/protein matches based on LC-MS/MS analysis of 15% SDS PAGE gels of eluates taken from IMHA patients. With the exception of complement C3 and immunoglobulin, many of these proteins are cytosolic or cytoskeletal in origin, indicating cytoplasmic matrix contamination of eluates.
<table>
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<tr>
<th>Band</th>
<th>Apparent mass on gel (kDa)</th>
<th>Predicted identity</th>
<th>Predicted molecular weight (kDa)</th>
<th>Number of unique peptides</th>
<th>Coverage (%)</th>
<th>Accession number (NCBI)</th>
<th>Probability of identity (%)</th>
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REFERENCES


APPENDICES

Appendix 1: Silver stain protocol modified from Shevchenko et al., 1996.

1. Fix gel in 50% methanol, 10% acetic acid in water for 2 x 20 minutes or overnight.
2. Wash for 10 minutes with 20% ethanol in MilliQ water.
3. Wash for 10 minutes with MilliQ water.
4. Sensitize the gel by treating for 1 min incubation in 0.02% sodium thiosulfate.
5. Rinse with two changes of distilled water for 1 minute each.
6. Incubate gel in 0.1% silver nitrate solution and agitate for 30 minutes at room temperature.
7. Rinse the stained gel twice with MilliQ water for 1 minute each.
8. Develop the gel in 0.04% formalin (37% formaldehyde in water) in 3% sodium carbonate and 10 mg/L sodium thiosulfate, with intensive shaking.
9. Replace with fresh developer once the developer has turned yellow.
10. Do not overdevelop the gels, stop the reaction in 1% acetic acid and store in acetic acid solution until analyzed.
Appendix 2: Protocol for 2D PAGE of erythrocyte lysates

Delipidation and protein precipitation (modified from Mastro and Hall, 1999 and Bruschi et al, 2005).

2. Add to clean glass tube 1 mL sample (aqueous homogenate of RBC ghosts) and 14 mL delipidizing solution, from step 1, such that acetone is 80% v/v (final).
3. Incubate at 4 degrees Celsius for 90 minutes.
4. Centrifuge at 2800 x g at 4 degrees C for 15-20 minutes.
5. Collect pelleted precipitate and wash sequentially in glass tubes (each step is followed by a centrifugation step, and the supernatant is discarded; Bruschi et al modify this by washing sequentially with the original delipidizing solution 3 times, instead of different fractions of it):
   a. 1 mL TBP
   b. 1 mL acetone
   c. 1 mL methanol
6. The pellet is then air-dried (allowing methanol and acetone to evaporate), before being rehydrated for isoelectric focusing.

Preparation of IPG strips and Isoelectric Focusing (based on Bruschi et al, 2005)

**Wear powder-free, clean nitrile gloves for all procedures!!**

1. Prepare focusing solution and store in 2.5 mL aliquots at -20 degrees Celsius until ready to use:
   a. 7 M urea
   b. 2 M thioruea
   c. 4% (w/v) CHAPS
   d. 5 mM tributylphosphine
   e. 20 mM iodoacetamide (IAA)
   f. 40 mM Tris
   g. 0.1 mM EDTA pH 8.5
   h. 0.5% IPG buffer, pH 3-10 (GE Healthcare, Baie d’Urfe, QC, Canada)
2. After delipidation of lysates is complete, incubate 30-50 ug of the sample with 2.5 mL focusing solution (step 1, above) for at least 3 hours at room temperature to allow proper reduction and alkylation of sulfhydryl groups.
3. To prevent over-alkylation, excess IAA is destroyed by adding equimolar amounts of dithiothreitol (DTT, 20 mM) to the tube, just prior to loading the ceramic strip holders.
4. Sample with focusing solution is carefully loaded into the strip holder (total = 250 uL for 13 cm immobilized pH gradient or IPG strip). Any bubbles left by pipetting can be removed by using a sterile hypodermic needle.
5. 13 cm IPG strip (Immobiline Dry Strip, GE Healthcare, Baie d’Urfe, QC, Canada) is slowly lowered (tapered anodic side first, toward corresponding tapered end of the ceramic strip holder) using capillary action (start at one end and lower progressively), being careful NOT to trap any air bubbles. Carefully lift and lower strip to ensure even wetting. Finally, the rectangular or cathodic end is lowered into the strip holder.

6. Apply 600 µL of IPG Cover Fluid (GE Healthcare) and place covers on strip holders.

7. Set strip holders in Ettan IPGphor Isoelectric Focusing Unit (GE Healthcare), making sure that each end is in the correct position and aligned along the gold electrodes.

8. Program unit:
   a. Rehydration = 12 hrs.
   b. Step 1 500V x 1 h = 500 V-h (step-n-hold)
   c. Step 2 1000V x 1 h = 1000 V-h (step-n-hold)
   d. Step 3 8000V x 2 h = 16,000 V-h (step-n-hold)

9. When finished running, the strips can be stored in sterile tubes at -70 degrees Celsius until further processing, or you may commence directly to second dimension (SDS PAGE). Protocol for SDS-PAGE of IPG strips used in this study is described in Harper et al, 1998 (pages 10.4.22 to 10.4.23).

**Fig. 8:** 2D PAGE of canine RBC membrane proteins based on protocol described in Appendix 2. Isoelectric focusing was performed on a 13 cm IPG strip pH 3-10 (Immobiline DryStrip, GE Healthcare); second dimension SDS-PAGE was performed using a 4-15% gradient gel on a Hoefer SE 600 Chroma electrophoresis unit (ThermoFisher Scientific, Waltham, MA, USA) at 200 mV constant for 4 hours.
Appendix 3: Solutions for Blue Native PAGE protocol

1. Sample Buffer
   a. 750 mM ε-aminocaproic acid
   b. 50 mM Bis-Tris HCl, pH 7.0
   c. 0.5 mM sodium EDTA

2. Digitonin solubilization buffer
   a. 30 mM HEPES pH 7.4
   b. 150 mM potassium acetate
   c. 10% (v/v) glycerol
   d. 5% (w/v) digitonin

3. Loading Buffer
   a. 750 mM ε-aminocaproic acid
   b. 5% (w/v) Coomassie G250

4. BN-electrophoresis running buffers
   a. Blue Cathode buffer (10x)
      i. 500 mM Tricine
      ii. 150 mM Bis-Tris HCl, pH 7.0
      iii. 0.2% Coomassie G250
   b. Colorless (clear) cathode buffer (10x)
      i. 500 mM Tricine
      ii. 150 mM Bis-Tris HCl, pH 7.0
   c. Anode buffer (10x)
      i. 500 mM Bis-Tris HCl, pH 7.0

5. Blue Native Gel Buffer (2x)
   a. 1 M ε-aminocaproic acid
   b. 0.1 Bis-Tris HCl, pH 7.0
Appendix 4: Solutions for immunoblot (western blot transfer solutions)

20x Stock transfer buffer
1. 200 mM Tris base 24.2 g
2. 2.0 M glycine 150.14 g
3. MilliQ water 1000 mL

10x Tris buffered saline (TBS) – pH adjusted to 7.5
1. Tris (THAM) 200 mM 24.2 g
2. NaCl 1.38 M 80.0 g
3. MilliQ water 1L

Blocking solution (1% bovine serum albumin in TBS)
1. Dilute 10x TBS: 50 mL of 10x stock TBS with 450 mL MilliQ water
2. Add: 0.5 mL Tween 20
3. Store: in 50 mL aliquots at -20°C

Washing buffer (Tween 20 TBS, pH 7.5)
1. Dilute 10x TBS stock: add 100 mL stock TBS with 900 mL MilliQ water
2. Add: 0.5 mL Tween 20
3. Adjust: pH to 7.5