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THE PREVALENCE AND SPECIFICITY OF ANTI-DOG ERYTHROCYTE ANTIGEN ANTIBODIES IN A POPULATION OF DOGS

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ABSTRACT

THE PREVALENCE AND SPECIFICITY OF ANTI-DOG ERYTHROCYTE ANTIGEN ANTIBODIES IN A POPULATION OF DOGS

By

Margaret Megan Lemmon

Antibodies to dog erythrocyte antigens (DEAs) have been reported to occur naturally in the dog population and can be induced after exposing a DEA-negative dog to the DEA. Anti-DEAs can cause transfusion reactions, including red cell loss and hemolytic transfusion reactions.

To determine the current prevalence of anti-DEAs in a population of dogs, 265 serum samples from dogs of unknown medical history were screened by the tube agglutination method with a red blood cell antigen panel representing all currently definable DEAs. Reactivity was demonstrated in 184 serum samples (69.4%) that were subsequently screened with a different red blood cell antigen panel to determine the specificity of the antibody or antibodies.

The prevalence of specific individual antibodies was: 1.9% anti-DEA 1.2, 5.3% anti-DEA 3, 3.0% anti-DEA 5, and 8.7% anti-DEA 7. These numbers do not reflect the antibodies associated with multiple antibody combinations.

Data were adjusted to exclude spurious results, and the prevalence was: 8.4% anti-DEA 3, 5.0% anti-DEA 5, 12.4% anti-DEA 7, 19.3% had possible multiple antibody combinations, and 14.8% were unknown antibodies.

This study indicated a higher overall prevalence of anti-DEAs than previous studies and a large percentage of the antibodies could not be defined. Consistent with previous studies, anti-DEA 3, 5, and 7 were identified.

DEDICATION

This Thesis is dedicated to my grandfather, William J. Barber, who taught me the value of continuing to learn no matter how old I am, and to my parents, D. Glenn and Margaret P. Lemmon, who have supported me in all of my endeavors.

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INTRODUCTION

Periodic surveys of American households conducted by the American Veterinary Medical Association (AVMA) have demonstrated the abundance of companion animals in America. During 1996, it was estimated that nearly 60 percent (%) of households in the United States had owned one or more pets, and out of these, nearly 32% of the households had owned dogs.[1] These figures reflect a 0.8% increase in the canine pet population since the AVMA survey conducted in 1991, which amounts to 0.4 million dogs.[1] With an increase in canine pet ownership, there is an increased need for veterinary care.[2,3]

In many cases, the companion animal is seen as a member of the family.[4,5] Given the importance of the pet's role in the family, many pet owners take steps to ensure the welfare of the animal through veterinary care. The increased demand for better pet health care has led to advancements in the quality and variety of services offered, including various surgical and medical procedures such as blood transfusions.[6,7] Despite these steps forward in veterinary medicine, the field lacks the regulations governing procedures such as blood transfusions that are a prominent feature of the human health care system. The failure to regulate such procedures leads to a lack of standardization in the field of veterinary medicine. At one end of the spectrum, a veterinarian may utilize techniques and protocols similar to those in human medicine, but on the other end, a veterinarian may do only what is necessary to get by. Standardization is essential to ensure quality health care for every animal.

The absence of standardization is especially significant in canine transfusion medicine. As opposed to human medicine, canine transfusion medicine is not subjected to regulations on the administration of blood products or pre-transfusion testing. Currently, a veterinarian may take a unit of blood from any dog and administer the unit to any recipient dog without pre-transfusion testing. This practice would be negligent in human medicine, but as there are currently no universal standards or regulatory guidelines in veterinary medicine, it is an accepted practice. Despite being within the limits of the law, the practice of this method of canine transfusion medicine is potentially risky. Without pre-transfusion testing, it is impossible to predict the likelihood of a transfusion reaction and may put the patient's life in jeopardy.

In most cases, canine transfusions are administered on an emergency basis, and complete pre-transfusion testing, including blood typing of the donor and recipient, serum antibody screening, infectious disease testing, and crossmatching is impossible. More in-house testing methods and a better understanding of the prevalence of antibodies against dog erythrocyte antigens (DEA) are needed to increase the safety of canine transfusion practices.

BACKGROUND

Immunohematology is the field involved with the interaction between immune factors and blood components. A major focus of immunohematology is the preparation of blood products for transfusion. Ideally, blood is collected from a healthy donor, typed, and compatible blood products are selected for transfusion into a recipient. Before the product is transfused into the recipient, a crossmatch is performed. The major crossmatch test involves combining the recipient's serum or plasma with the donor's red blood cells to detect the presence of circulating antibodies in the recipient against antigens on the donor's red blood cells. The donor's serum or plasma can also be screened for antibodies against the recipient's erythrocyte antigens in a minor crossmatch. Following testing, units of blood are selected for transfusion by matching the antigenic profile of the donor's red blood cells to the antigenic profile of the recipient's red blood cells. When units of blood cannot be matched to the recipient's antigen profile, units typed as antigen-negative may be selected for transfusion.

Immunohematology

Antigens are defined as substances that can induce antibody synthesis.[8,9] More specifically, antigens can be composed of proteins, carbohydrates, or lipids. Canine blood group antigens have not been fully characterized for biochemical composition, although they may be comprised of glycoproteins or glycolipids.[9]

Antibodies are immunoglobulins (Ig) that exhibit specificity for particular antigens. There are 5 classes of immunoglobulin in dogs: IgA, IgG, IgM, IgD, and IgE.[8] The distinctions between the classes of immunoglobulin are the result of differences in the constant region of the heavy chain, which does not convey specificity. Rather than resulting in varied specificity, differences in immunoglobulin structure are responsible for varied function. Immunoglobulins also have varied effects depending on whether they are *in vivo* or *in vitro*. Immunoglobulins bound *in vivo* can result in complement activation, opsonization, neutralization, and rarely, agglutination, while immunoglobulins bound *in vitro* may result in complement activation and agglutination.[8,9]

The surface of the red blood cell is covered with antigens, which give the cell its immunological identity. Plasma contains antibodies, which can be "naturally-occurring" or acquired. "Naturally-occurring", or expected, antibodies occur without exposure to other blood types and are present in the blood from shortly after the time of birth. Acquired antibodies are produced as a result of exposure of an antigen-negative patient to that antigen.[10] Exposure can occur when transfused red blood cells that are recognized as foreign induce the production of antibodies against the foreign antigens in a process called red blood cell alloimmunization.[11] Exposure may also occur during pregnancy or during delivery in an antigen-negative mother carrying an antigen-positive fetus.[11-15]

During allogeneic red blood cell transfusion, immunologic reactions due to antibodies may occur. Such reactions can be immediate or delayed, with

symptoms appearing during or shortly after transfusion for immediate transfusion reactions or to approximately 21 days post-transfusion for delayed transfusion reactions, although symptoms may occur later.[16] The reaction could be acute and threaten the survival of the transfusion recipient, or may lack physical manifestation. At the cellular level, immunologic reactions to transfusion are the result of complement activation or opsonization by complement and/or immunoglobulin.[8,11,16,17] This process of transfused red blood cell clearance is called immune-mediated red blood cell destruction.[11,16]

Antigens on the surface of transfused red blood cells can bind to B cell receptors, which are membrane-bound immunoglobulins.[8,11] Once an antigen binds to the receptor, a signal is transmitted to the B cell interior and the antigen is endocytosed by the B cell. The antigen is degraded and displayed on the surface of the B cell as peptides on major histocompatibility complex (MHC) class II molecules. When a T helper cell of the same antigen specificity recognizes the peptide-MHC class II complex, the T cell is activated, begins expressing the CD40 ligand on its surface, and secretes interleukin (IL) 4, IL-5, and IL-6, which are responsible for stimulating the B cell to proliferate and differentiate into plasma cells. The plasma cells are responsible for antibody production of the same specificity as the B cell receptor.[11]

Macrophages can also bind the constant region of immunoglobulins bound to surface antigens of red blood cells, and subsequently phagocytose the cell. The macrophage degrades the red blood cell into end products including peptides. The peptides can be released outside of the macrophage or they can

be displayed on MHC class II molecules on the macrophage cell surface, converting the macrophage into an antigen presenting cell. The released peptides can interact with B cells, leading to B cell proliferation and differentiation. The macrophage acting as an antigen presenting cell can activate naïve T helper cells with the peptide-MHC class II complex. Activated T helper cells can recognize peptide-MHC class II complexes on activated B cells, leading to antibody production.[8]

Immune-mediated red blood cell destruction is based on either complement (C) activation, opsonization, or a combination of both complement activation and opsonization. There are two principal ways these conditions can lead to the destruction of red blood cells: intravascular hemolysis and extravascular red blood cell destruction. Intravascular hemolysis involves complement activation leading to the activation of the membrane attack complex. The cell membrane integrity is compromised and the cell is destroyed.[11]

Immune-mediated red blood cell destruction by extravascular mechanisms can occur in three different ways: C3b alone, Ig alone, or C3b and Ig together. The mechanism of C3b alone is relatively ineffective in causing red blood cell destruction. Red blood cells with C3b present on the cell surface may adhere to phagocytic cells, although there is usually minimal phagocytosis of these cells because C3b alone does not stimulate phagocytosis.[11] C3b can lead to phagocytosis when macrophage-activating immune mediators, such as C5a, are present. Often, the C3b bound to the cell surface is converted to C3d by enzymatic cleavage, and the red blood cell can usually survive normally. Red

blood cells with IgG present on the cell surface usually bind to the Fc receptors of phagocytic cells and subsequently are phagocytosed. Red blood cells with both C3b and IgG present on the cell surface are more successfully destroyed than red blood cells with either C3b or IgG alone because they can more readily adhere to macrophages and induce phagocytosis.[6,8,9,11,18]

Systemic manifestations of transfusion incompatibility can be highly varied, depending on the agents involved in the event. The most severe hemolytic transfusion reactions are the result of preexisting antibodies in the recipient interacting with antigens on transfused red blood cells.[19] The antigen-antibody interaction can lead to a series of events including complement activation, cytokine production, coagulation activation, and other systemic inflammatory responses. Complement activation can cause intravascular hemolysis resulting in hemoglobinemia and possibly hemoglobinuria. The anaphylatoxins produced as a result of complement activation can cause hypotension and bronchospasm, and can lead to the release or production of systemic or local mediators, such as histamine, kinins, and cytokines. The mediators may lead to physical manifestations that are similar to those of systemic allergy, such as flushing, chest pain, and vomiting.[16]

The full role of cytokines in the manifestations of immune-mediated hemolysis is not completely known, although certain cytokines are known to lead to fever and hypotension, and stimulate endothelial cells to increase procoagulant activity. The antigen-antibody interaction may also have a role in coagulation activation by initiating the "intrinsic" pathway through the Hageman

factor. Activated Hageman factor leads to a series of processes that increase the expression of tissue factor.[11] The presence of tissue factor activates the "extrinsic" coagulation pathway and is associated with disseminated intravascular coagulation.[11] The outcome of these events may be uncontrolled bleeding or oozing.[11] Other systemic manifestations, such as renal failure and shock, result from the mass of antigen-antibody complexes, immune mediators, and systemic effects resulting from complement activation, cytokine production, and coagulation activation.[11,20-23]

The History of the Dog Blood Group System

Red blood cell antigens were first discovered in humans in 1900 by Landsteiner.[24] This discovery sparked the search for similar blood antigen systems in other species, and in 1910, Von Dungern and Hirszfeld made the discovery of dog blood group antigens.[22] Four different antigens were described at that point. In the 1950s and 1960s, the most extensive work in dog blood group research was conducted and reviewed by Swisher and Young.[12,13,18,25] This work described seven blood group antigens in dogs and the frequency of each antigen within a random population of dogs. The study also focused on antibodies specific for the blood group antigens.[13]

The Dog Blood Group System

The dog blood group system is based on the antigens found on the surface of the dog red blood cell. Some of these antigens have been identified

serologically, but the structures have not been well-elucidated.[6,12,26] The First and Second International Workshops on Canine Immunogenetics standardized the terminology of the blood group system.[27,28] Although most dog immunohematology professionals adhere to the standardized terminology, some groups, including the dog immunohematology community in Japan – which was not a part of the International Workshops - do not adhere to the standard terminology.[27-30] As decided at the First International Workshop, the red blood cell antigens were called canine erythrocyte antigens, or CEAs, which was changed to dog erythrocyte antigens, or DEAs, at the Second International Workshop.[27,28] Each antigen is identified by the abbreviation DEA followed by a number indicating the blood group. In the case of subgroups, the antigen is identified by the term DEA followed by the blood group number, a period, and the number of the subgroup. An exception to this rule exists in the case of DEA 7; letters rather than numbers specify the subgroups. Although many blood groups have been identified, currently five blood groups are of clinical significance, which means they can be routinely identified by serologic methods and can potentially cause a stimulating event after transfusion into an antigen-negative dog. The current clinically significant groups are DEA 1, 3, 4, 5, and 7. The only clinically significant groups to have subgroups are DEA 1 and DEA 7.

The DEA 1 group consists of four phenotypes: DEA 1.1, 1.2, 1.3, and null.[31] Only one DEA 1 group phenotype can be expressed in any particular dog. A pattern of autosomal dominance is demonstrated in the DEA 1 group with the order of descending dominance as follows: DEA 1.1, DEA 1.2, DEA 1.3, and

null.[28,32] The DEA 1.1 phenotype is expressed in about 42% of the general population and DEA 1.2 is expressed in about 20% of the general population, although variation in expression occurs in different breeds.[12,33] Greyhound and German Shepard breeds are typically DEA 1.1 and 1.2 negative, whereas Golden Retriever, Laborador, and Rottweiller breeds are usually DEA 1.1 or 1.2 positive.[33] No estimate of the frequency of DEA 1.3 has been reported. DEA 1.1 and 1.2 are consistently recognized by commercially-available antisera, but DEA 1.3 may be missed with the same antisera.[34] The DEA 1 group is of particular importance due to its frequency and its ability to induce antibody production in DEA 1-negative dog.[13,15,21,35] Membrane proteins with molecular weights of 50 and 200 kilo Daltons (kD) have been identified for DEA 1.1 in Western blot experiments utilizing an anti-DEA 1.1 monoclonal antibody.[36] An 85 kD band has been identified for DEA 1.2 in immunoprecipitation experiments utilizing polyclonal antibodies.[26]

The DEA 3 group consists of two phenotypes: DEA 3 and null.[31] Autosomal dominance is exhibited for this group with DEA 3 dominant over the null phenotype.[32] The DEA 3 phenotype shows breed specificity, being expressed in up to 23% of greyhounds but only 6% in the general population.[12] Up to 20% of DEA 3-negative dogs may have a naturally occurring anti-DEA 3 antibody.[12,15] DEA 3-negative dogs that have been sensitized to the DEA 3 antigen may suffer severe transfusion reactions upon subsequent exposure to DEA 3-positive red blood cells. DEA 3-positive red blood cells repeatedly transfused to a previously sensitized DEA 3-negative dog could result in cell loss

within a period of five days.[14,30] Five bands have been identified in Western blotting experiments utilizing an anti-DEA 3 monoclonal antibody. The molecular weights of the bands were 34, 53, 59, 64, and 71 kD.[37]

The DEA 4 group consists of two phenotypes: DEA 4 and null.[31] This group exhibits autosomal dominance, with DEA 4 dominant over the null phenotype.[32] Nearly all dogs (98-99%) express DEA 4, although variance may occur among specific breeds and with variation in geographic location.[12] No naturally occurring antibody against DEA 4 has been reported. Red blood cells positive for DEA 4 only are the universal blood type and are usually considered safe to transfuse into dogs of other DEA types. An early study by Swisher et al. (1962) in which DEA 4-negative dogs were exposed to DEA 4-positive cells demonstrated that anti-DEA 4 antibodies were produced in response to the exposure, but subsequent exposure to DEA 4 did not result in transfusion reactions.[14] A more recent study by Melzer et al. (2003), however, demonstrated a severe transfusion reaction in a DEA 4-negative dog that had been administered multiple units of DEA 4-positive blood.[38] A protein of molecular weight between 32 and 40 kD has been isolated using polyclonal anti-DEA 4 antibodies in immunoprecipitation experiments.[26]

The DEA 5 group has two phenotypes: DEA 5 and null.[31] Autosomal dominance is exhibited in the DEA 5 group, with DEA 5 dominant over the null phenotype.[32] The DEA 5 phenotype shows breed specificity, being expressed in up to 23% of the general population but up to 30% in greyhounds.[12] Variance may also occur due to geographical location, as with the human Duffy

antigen.[12,39] The majority of dogs within a geographical area, thus within the potential breeding population, usually remain within that location, and the antigen or its absence also remains as a characteristic within the location. Previous studies have reported naturally occurring anti-DEA 5 antibody in approximately 10% of randomly-selected non-transfused adult dogs in the United States.[14,15] Upon repeated exposure to DEA 5-positive red blood cells, DEA 5-negative dogs sensitized to DEA 5 can sequester and destroy the transfused red blood cells within a period of three days.[14] The DEA 5 antigen has not been characterized.

The DEA 7 group has three phenotypes: DEA Tr, O, and null.[31] This is the only blood group that does not obey the rules of DEA nomenclature. Although DEA 7 has multiple phenotypes, the subgroups are not usually indicated individually in studies or in blood typing by reference laboratories. Unlike the other DEAs, DEA 7 is not a true erythrocyte antigen.[40] Production of this antigen occurs in the body tissues and the soluble antigen is absorbed onto the surface of the red blood cell, similar to human blood group antigen A.[31,41-43] The DEA 7 blood group is expressed in about 45% of the general population.[12] Previous studies demonstrated an prevalence of anti-DEA 7 antibody in up to 50% of dogs that were negative for the DEA 7 antigen.[12,44,45] DEA 7-negative dogs may be sensitized to DEA 7 through exposure to the antigen on red blood cells, and repeated exposure to the antigen in transfusion may result in sequestration of the red blood cells and cell loss within a period of 72 hours.[12] Three bands of the molecular weights 53, 58, and 66 kD have been isolated by immunoprecipitation experiments utilizing polyclonal anti-DEA 7 antisera.[26]

"Naturally-occurring" anti-DEA antibodies have been demonstrated against DEAs 3, 5, and 7.[14,15,46] Many researchers disagree how frequently these antibodies occur, especially with anti-DEA 7.[12,21,24,34] Hale (1995) reported an prevalence of the anti-DEA 7 antibody in up to 50% of DEA 7 negative dogs, although the author's personal observation suggested an prevalence of 20% to 50%.[12] Other reports indicate an prevalence of anti-DEA 7 in 15% to 50% of DEA 7 negative dogs. Some early researchers doubted the existence of anti-DEA antibodies altogether.[47,48] More recently, Giger et al. (1995) suggested that dogs do not have naturally occurring antibodies of clinical significance as are present in cats and humans, which have antibodies that are expected and can cause severe hemolytic transfusion reactions.[21] Today, the existence of anti-DEA antibodies is generally accepted, but their significance in dog transfusion medicine may still be debated.[21,35,49,50]

Currently, there are no standards or regulatory guidelines for transfusion practices in veterinary medicine. At the discretion of the veterinarian, testing can be extensive or may not be done at all.[47] Although no regulatory guidelines exist, many methods are available for pre-transfusion testing, including blood typing of the donor and recipient by serologic methods, major and minor crossmatching, rapid agglutination card tests, and, more recently, the gel tube crossmatching kit.

Description of Pre-transfusion Testing Methodologies

The serologic method of blood typing relies on tube agglutination to determine the presence of DEAs.[51] The technique involves combining polyclonal anti-DEA antisera with a suspension of washed red blood cells to be typed, followed by an incubation period at an appropriate temperature: 37 degrees Celsius (°C) for anti-DEA 1.X and anti-DEA 1.1, and 4°C for anti-DEA 3, anti-DEA 4, anti-DEA 5, and anti-DEA 7. A negative control reaction is run at both temperatures, as well, with PBS in place of the antisera. After incubation, the samples are centrifuged and the cells are gently resuspended while being viewed for agglutination. Presence of agglutinates indicates the presence of the DEA. Blood typing by serologic methods requires the skill of specially-trained technicians and uses expensive reagents. As a result, reference laboratories usually conduct this method of typing.[9,12,51,52] Commonly, two versions of serologic typing are available: full DEA typing and abbreviated DEA typing. The full typing involves the use of all currently available anti-DEA antisera: anti-DEA 1.X, anti-DEA 1.1, anti-DEA 3, anti-DEA 4, anti-DEA 5, and anti-DEA 7, as well as anti-canine globulin (ACG).[53] Full typing tests for all clinically-significant DEAs and is, therefore, the most extensive dog blood typing procedure. Some abbreviated typing panels use anti-DEA 1.X, anti-DEA 1.1, and anti-DEA 7, while others may only use anti-DEA 1.1 and anti-DEA 1.X. The corresponding antigens – DEA 1.1, 1.2, 1.3, and 7 – are of significant interest to the veterinarian because of their high prevalence in the dog population and potential for stimulating antibody production.[12] This typing paradigm, however, excludes

testing for DEA 3, 4, and 5, which all have the potential to cause transfusion reactions. [12,38] There are also known naturally-occurring serum antibodies to DEA 3 and 5, which can cause transfusion reactions, further emphasizing the importance of these DEAs in pre-transfusion testing.

The crossmatch procedure is used to detect incompatibility between the blood donor and the recipient of the blood component.[9,52] This technique relies on the combination of red blood cells with serum in a tube agglutination method. As with the serologic blood typing method, the samples are incubated at various temperatures, centrifuged, and gently resuspended while being viewed for agglutination. The presence of agglutinates indicates an incompatibility between the donor and recipient. Two variations of the crossmatch may be performed: major and minor. In a major crossmatch, donor red blood cells are combined with recipient serum, which is intended to simulate the reactions that may occur when the recipient receives the red blood cell component in a transfusion. In a minor crossmatch, donor serum is combined with recipient red blood cells, which is intended to simulate the reactions that may occur when the recipient receives the plasma component in a transfusion. Controls may or may not be performed depending on the standard operating procedures of the individual laboratory or clinic, although the inclusion of controls would ensure that the test is running properly and that a positive crossmatch is not due to autoagglutination. A negative control for the crossmatch procedure would consist of running the reaction as described but using PBS in place of the serum. A recipient receiving

whole blood would benefit from both the major and minor crossmatches, although the minor crossmatch is less frequently performed in practice.

The gel tube major crossmatch kit, which is based on the principles of the tube agglutination crossmatch procedure, is a very recent addition to the canine pre-transfusion testing options. It consists of a sepharose gel matrix in a microcentrifuge tube (Figure 1). The test is performed by combining donor red blood cells with recipient serum and placing the mixture on top of the gel matrix. The tube is centrifuged and viewed for agglutinates. A positive test will show the presence of agglutinates at the top of the gel matrix. A negative test will show no agglutinates and the red blood cells will be collected at the bottom of the tube. Positive and negative control reactions are included with the kit. This test is inexpensive and very easy to perform, making it an affordable pre-transfusion testing method for privately-owned veterinary clinics.

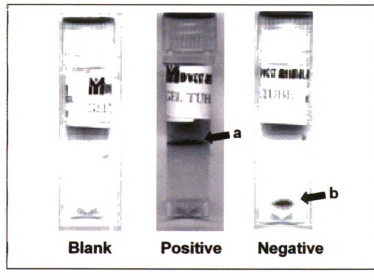

Figure 1: Gel Tube Major Crossmatch Test. A positive reaction is demonstrated by the presence of agglutinates at the top surface of the gel column (a). A negative reaction is demonstrated by the accumulation of non-agglutinating cells at the bottom of the gel column (b). (Picture from Midwest Animal Blood Services, Inc.)

The rapid agglutination card test is a commercially available assay for the detection of DEA 1.1. The test consists of a murine monoclonal anti-DEA 1.1 antibody that is lyophilized onto the card.[51,54] The red blood cells to be typed are mixed with the antibody on the card and the card is viewed for agglutination. The presence of agglutinates indicates the presence of DEA 1.1 (Figure 2). Card tests may or may not include control spots. The Rapid Vet-H DEA 1.1 test includes both positive and negative control wells.[54] This test is also inexpensive and easy to use, which makes it a reasonable option for veterinarians to perform in their clinics. This test lacks the ability to detect the other DEA 1 subgroups – DEA 1.2 and 1.3 – and no other DEA types can be tested by this method because there have not been any successful versions produced other than DEA 1.1.

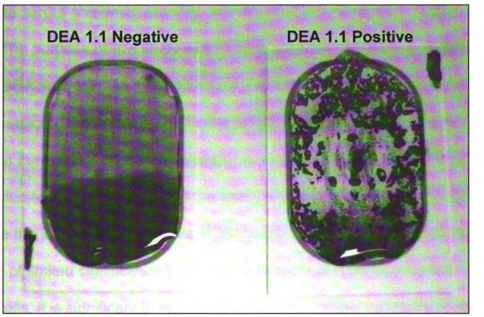

Figure 2: DEA 1.1 Card Test. A negative reaction is demonstrated by a uniform suspension of cells and the absence of agglutinates. A positive reaction is demonstrated by the presence of agglutinates.

Previous studies focused on determining the prevalence of anti-DEA antibodies in dog serum are informative, yet were conducted at limited reaction conditions and reflect a population of dogs that existed more than 50 years ago. In the study by Young et al. (1952), a population of only 145 dogs were screened and the testing was conducted only at room temperature.[15] Additionally, the study counted as positive only those reactions that were of 1+ strength and greater, and could not be "dispersed with moderate agitation".[15] Changes in veterinary medical practices, including the increase in blood transfusions, may have an effect on the prevalence of acquired anti-DEA antibodies in the current dog population. Anti-DEA antibody prevalence and distribution may also have also been impacted by changes in breeding practices. This study was conducted with a current population of dogs, a greater number of serum samples, a more complete serum screening process, and different temperatures.

The purpose of this study was to determine the prevalence and specificity of anti-DEA antibodies in a population of dog serum samples. Some previous studies indicate that naturally-occurring anti-DEAs are not clinically-significant because they do not cause hemolysis or hemolytic transfusion reactions.[46,51] Other studies have demonstrated the ability of naturally-occurring anti-DEAs to mediate the loss of red blood cells.[12] While the loss of transfused red blood cells may be of limited significance in recipients capable of regenerating their own red blood cells, it is significant in recipients with compromised capability of red blood cell regeneration. The loss of red blood cells in a recipient incapable of red blood cell regeneration will likely lead to a need for subsequent transfusions. Acquired

anti-DEA antibodies are a concern because, unlike naturally-occurring anti-DEAs, acquired antibodies may have hemolytic properties and may lead to more severe transfusion reactions.[12,51,52]

METHODS

Selection of Screening Red Blood Cells

The selection of canine blood samples was based on DEA profile. The Antibody Detection Screening panel consisted of three dogs of the following antigenic profiles: DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, and DEA 1.3,4,w7 positive. These dogs were selected to represent all clinically-significant DEAs. The Antibody Identification Screening panel consisted of four dogs of the following antigenic profiles: DEA 1.1,4,5,7 positive, DEA 3,4,5 positive, DEA 1.1 positive, and DEA negative. These dogs were selected to allow for identification of the antibodies detected in the antibody detection panel by exclusion; a serum sample that did not react with a particular red cell would not be considered as containing antibodies to the DEAs represented on that red cell (Figure 3). Additionally, dogs were selected based on the probability value calculations by the Fisher Exact and the Harris and Hochman methods.[55] The dog blood donors selected provide a probability (p) value of less than 0.05 for being able to identify all DEAs except DEA 1.2 and DEA 1.3. There were insufficient red blood cell donors available to give a p-value of less than 0.05 for being able to identify DEA 1.2 and 1.3. These two DEAs had a p-value of 0.147 by the Fisher method and 0.057 by the Harris and Hochman method (Table 1).

	Screen Cell	DEA							Set 1			Set 2		
		1.1	1.2	1.3	3	4	5	7	IS	RT	4°C	37°C	ACG	P
Detection Screen	Pearl	+	0	0	+	+	+	+						
	Emily	0	0	+	0	+	0	w+						
	Skipper	0	+	0	0	+	0	+						
Identification Screen	Sara	+	0	0	0	0	0	0						
	Dillon	+	0	0	0	+	+	+						
	Emma	0	0	0	0	0	0	0						
	Boo Boo	0	0	0	+	+	+	0						

Figure 3: Antibody screening panel. The red blood cell samples were selected based on DEA type to allow for the detection and identification of antibodies in the serum samples. The Detection Screen represented all clinically significant DEAs. The Identification Screen contained DEA types to allow for the identification of the antibody type by exclusion. Set 1 represented cold reactions. Set 2 represented warm reactions. w+ = weak positive, IS = immediate spin, RT = room temperature, ACG = anti-canine globulin, P = positive.

Table 1: Probability values for each DEA based on the Fisher Exact and the Harris and Hochman (H&H) methods

DEA	# Positive	# Negative	p-value (Fisher)	p-value (H&H)
1.1	3	4	0.029	0.008
1.2	1	6	0.143	0.057
1.3	1	6	0.143	0.057
3	2	5	0.048	0.015
4	5	2	0.048	0.015
5	3	4	0.029	0.008
7	4	3	0.029	0.008

Statistics

The calculation of the p-value was conducted for DEAs 1.1, 1.2, 1.3, 3, 4, 5, and 7. Two methods were used to calculate the p-values for each DEA. The first method, the Fisher Exact method, is the traditional method of calculating the probability that the correct antibody was identified.[55] This method compares the number of screening cells demonstrating a particular antigen with the number of screening cells lacking the antigen, to give a p-value. The Fisher's exact method formula is:

$$\frac{(A+B)! \times (C+D)! \times (A+C)! \times (B+D)!}{N! \times A! \times B! \times C! \times D!}$$

A = number of positive reactions observed with DEA-positive RBCs

B = number of positive reactions observed with DEA-negative RBCs

C = number of negative reactions observed with DEA-positive RBCs

D = number of negative reactions observed with DEA-negative RBCs

N = number of RBCs tested

For example, if three DEA 5 positive dogs and four DEA 5 negative dogs were used to screen serum samples, and assuming no false positive or false negative reactions were observed, the formula would look like:

$$\frac{(3+0)! \times (0+4)! \times (3+0)! \times (0+4)!}{7! \times 3! \times 0! \times 0! \times 4!}$$

This formula would give a p-value of 0.029.

The second method, the Harris and Hochman method, is a more liberal calculation of p-value, and is becoming more widely accepted for antibody probability calculation.[55] The equation for the Harris and Hochman method is:

$$(A/N)^A(B/N)^B$$

A = number of positive reactions observed with DEA-positive RBCs

B = number of negative reactions observed with DEA-negative RBCs

N = number of RBCs tested

For example, if three DEA 5 positive dogs and four DEA 5 negative dogs were used to screen serum samples, the formula would look like:

$$(3/7)^3 \times (4/7)^4$$

This formula would give a p-value of 0.008.

A p-value of 0.05 or less is considered significant as it suggests that there is a 5% or less chance a sample could give the specified reaction pattern and have a specificity other than the defined specificity.[55]

Blood collection and storage

Using the Vacutainer™ (Becton-Dickinson) blood collection system, blood was collected via venipuncture from dogs in colony at Michigan State University (MSU) in tubes containing acid citrate dextrose (ACD) and from dogs in colony at Midwest Animal Blood Services, Inc. (MABS) in tubes containing tri-potassium ethylene diamine tetra-acetic acid (K_3EDTA). Collected blood was transferred to 15 milliliter (ml) conical screw-cap tubes for storage. Samples collected from MABS also had AS-5 Optisol ® Red Cell Preservative Solution (Terumo Corporation, Tokyo, Japan) added to the tube. Alsever's solution (See Appendix) was added to all samples in a ratio of 2 parts Alsever's solution to 7 parts blood to extend cell lifespan. Blood was stored at 4°C for up to 34 days, although most samples were used within 15 days.

Serum samples

Dog serum samples were obtained from MABS. Serum samples were submitted to MABS between 1995 and 2000 for routine serum antibody screening and were heat inactivated at 56°C for 30 minutes (min) as per standard operating procedure at MABS. Most samples were from dogs that were potential red blood cell or plasma donors, and therefore the samples may closely reflect the donor pool. The samples, however, may not reflect the recipient pool, as it may not necessarily include dogs excluded by donation criteria, such as dogs under 50 pounds or dogs over 7 years old. More extensive patient histories of these dogs, including history of pregnancy or transfusion, is unknown. The

samples were stored in microcentrifuge tubes at –70°C. Samples with a volume of 0.9 ml or greater were included in the study, for a total of 312 samples studied.

Cell washing

Samples of 1 ml of whole dog blood at 4°C were dispensed into 12 x 75 millimeter (mm) borosilicate glass disposable culture tubes and phosphate buffered saline (PBS) (See Appendix) at room temperature (RT) was added to approximately 1 centimeter (cm) below the opening of the tubes. The tubes were centrifuged for 2 min at 1140 gravities (x g) in an Immufuge II (Baxter Healthcare Corporation, Deerfield, IL). The supernatant was vacuum-aspirated. The remaining cells were resuspended in fresh PBS, centrifuged, and aspirated two additional times to yield washed packed red blood cells.

Sera screening

Serum screening was conducted between March 2003 and October 2005. The Detection Screen was completed between March 2003 and March 2004. The Identification Screen was completed in October 2005.

For the Detection Screen, six 10 x 75 mm borosilicate glass disposable culture tubes were labeled with the identification number for each serum sample. These tubes were divided into sets of two tubes, which were additionally labeled to correspond with each of the three red blood cell samples. One tube from each set of two was designated as a 37°C reaction tube. The other tube was designated as a 4°C reaction tube.

For the Identification Screen, each serum sample that demonstrated a positive reaction in the first panel was rescreened. Eight 10 x 75 mm borosilicate glass disposable culture tubes were labeled with the identification number for each serum sample. These tubes were divided into sets of two tubes, which were additionally labeled with each red blood cell donor's name. One tube from each set of two was designated as a 37°C reaction tube. The other tube was designated as a 4°C reaction tube.

A volume of 30 microliters (μl) of washed packed red blood cells was thoroughly resuspended in 970 μl of PBS to prepare a 3% red blood cell suspension for each red blood cell sample.

Each serum sample to be screened was thawed and centrifuged at 5500 x g for 5 min to remove particulate matter. Fifty μl of the serum sample was dispensed into the 10 x 75 mm borosilicate glass tubes labeled with the serum sample identification number. Fifty μl of the appropriate 3% red blood cell suspension was added to each tube. (Figure 4)

To detect the presence of anti-DEA antibody, each serum sample was exposed to two different sets of reaction conditions. The first set of reaction conditions involved an immediate spin followed by a 30 min incubation at RT followed by a 30 min incubation at 4°C (Figure 4). After the initial addition of the 3% red blood cell suspension to the serum samples and after each incubation step, the tubes were centrifuged for 15 seconds (s) at 1140 x g and the supernatant was viewed for hemolysis. The cell button was gently resuspended and viewed for agglutination using an Agglutination Viewer (Clay-Adams).

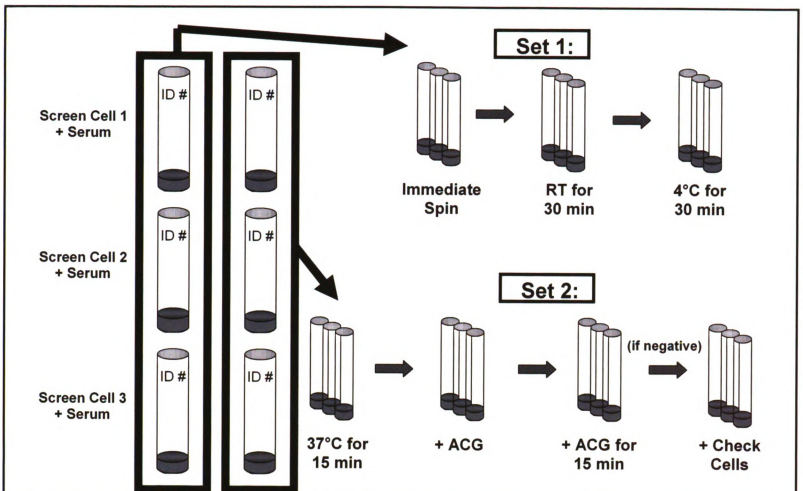

Figure 4: Reaction set-up. Serum sample is added to 6 tubes labeled with the identification number. Screen Cell 1 is added to 2 of the tubes, Screen Cell 2 is added to 2 of the tubes, and Screen Cell 3 is added to 2 of the tubes. A set consists of 1 tube with Screen Cell 1, 1 tube with Screen Cell 2, and 1 tube with Screen Cell 3. Set 1 is viewed after Immediate Spin, Room Temperature (RT) for 30 minutes, and 4°C for 30 minutes. Set 2 was viewed after 37°C for 15 minutes, ACG, ACG for 15 minutes, and Check Cells.

Reactions were graded based on the American Association of Blood Bank Technical Manual standards. Figure 5 depicts the reaction grades and describes the corresponding reaction characteristics. A positive reaction was defined as a reaction with strength of 1+ or greater, and a negative reaction was defined as trace or 0. A trace reaction was considered negative because it consisted of mainly of uniformly resuspended red blood cells with only a few tiny agglutinates.

The second set of reaction conditions included a 15 min incubation at 37°C followed by three cell washes with PBS, the addition of ACG reagent (See Appendix), and a 15 min incubation at room temperature with ACG (Figure 4). Following the 15 min incubation at 37°C, the ACG addition, and the 15 min incubation with ACG, the tubes were centrifuged and the supernatant was viewed for hemolysis. The cell button was gently resuspended and viewed for agglutination. For each sample exhibiting a negative reaction, check cells (See Appendix) were added and the tube was centrifuged and viewed for agglutination. A mixed field reaction, which is characterized by many agglutinates in an even resuspension of unbound red blood cells, indicated the ACG was reacting properly.

Positive and negative controls were run during each day of screening to demonstrate the viability of the red blood cells used and to ensure the test was within specifications. The number of batches run per day varied from 1 batch of 8 samples to 5 batches of 8 samples each, but only one set of controls was run on each day regardless of the number of batches run. For each control in the first panel, six 10 x 75 mm borosilicate glass disposable culture tubes were

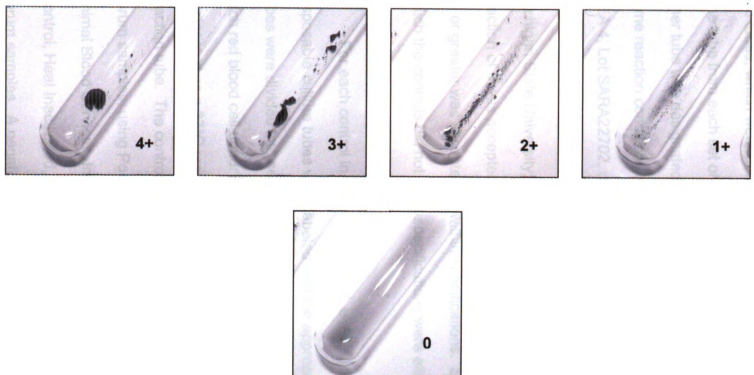

Figure 5: Reaction grades. A 4+ reaction is characterized by one large agglutinate and clear supernatant. A 3+ reaction is characterized by several large agglutinates and clear supernatant. A 2+ reaction is characterized by many medium-sized agglutinates. A 1+ reaction is characterized by many small-sized agglutinates. A 0 reaction characterized by the absence of agglutinates and a uniform suspension of red blood cells. A trace reaction (not pictured) is characterized as a uniform suspension of red blood cells with only a few tiny agglutinates.

labeled with the appropriate control name. These tubes were divided into sets of two tubes, which were additionally labeled with each red blood cell donor's name. One tube from each set of two was designated as a 37°C reaction tube. The other tube was designated as a 4°C reaction tube. The controls were run at the same reaction conditions as the serum samples, using Positive Control I (Anti-DEA 4, Lot SARA22702, diluted 1:25, Midwest Animal Blood Services, Inc.) and Negative Control Serum (Canine Negative Control, Heat Inactivated, Pooled, Michigan State University) in place of the serum samples. A negative control reaction of 0 was accepted as within specifications. A positive control reaction of 2+ or greater was accepted as within specifications. Samples run on a day in which the controls did not meet specifications were either rescreened or removed from the data set.

For each control in the second panel, eight 10 x 75 mm borosilicate glass disposable culture tubes were labeled with the appropriate control name. These tubes were divided into sets of two tubes, which were additionally labeled with each red blood cell donor's name. One tube from each set of two was designated as a 37°C reaction tube. The other tube was designated as a 4°C reaction tube. The controls were run at the same reaction conditions as the serum samples, using Positive Control II (Goat Anti-Dog, Lot G9-3-8-05, Midwest Animal Blood Services, Inc.) and Negative Control Serum (Canine Negative Control, Heat Inactivated, Pooled, Michigan State University) in place of the serum samples. A negative control reaction of 0 was accepted as within specifications. A positive control reaction of 2+ or greater was accepted as within

specifications. Samples run on a day in which the controls did not meet specifications were either rescreened or removed from the data set.

RESULTS

In this study, serum samples were screened to detect the presence of antibody against any clinically significant DEA and samples demonstrating positive reactions were rescreened to identify the specificity of the reacting antibody. Data were generated for 312 serum samples. A total of 47 serum samples were excluded due to a day of failed negative control (10 samples), failed check cells (10 samples), a clerical discrepancy (16 samples), technical error (8 samples), and non-specific complete hemolysis (3 samples). Data are presented for a total of 265 serum samples (Table 2).

Detection Screen

The Detection screening was conducted using dogs with the antigenic profiles of DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, and DEA 1.3,4,w7 positive, as previously described. This screening resulted in 81 negative reactions (30.6%) (Table 3). Of the remaining 184 samples, a total of 125 samples (47.2%) showed positive reactions at both RT/4°C (cold) and 37°C /ACG (warm) conditions, 47 samples (17.7%) were positive only at cold conditions, and 12 samples (4.5%) were positive only at warm conditions.

Table 3: Summary of reaction conditions in the Detection Screen

Reaction Pattern	Number of Samples	Percentage of Total (%)
Negative	81	30.6%
Positive cold & warm	125	47.2%
Positive cold, Negative warm	47	17.7%
Negative cold, Positive warm	12	4.5%
Total	265	100%

Table 2: Detailed combined results of the Detection and Identification Screens

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
6934	0	0	0	0	0	0								
6935	3+	0	0	0	0	0	0	2+	0	0	0	tr	0	0
Buddy	4+	0	0	4+	0	2+	0	3+	0	0	0	2+	0	0
C188	3+	2+	2+	2+	0	0	0	0	0	3+	0	0	0	1+
M6965	4+	2+	1+	2+	1+	1+	0	1+	0	0	0	0	0	0
M6966	4+	0	0	4+	0	1+	0	0	0	0	0	0	0	0
M6967	0	0	0	0	0	0								
M6968	3+	0	0	1+	0	0	0	0	0	0	0	0	0	0
M6969	4+	0	0	3+	0	0	0	0	0	0	0	0	0	0
M6970	0	0	0	0	0	0								
M6971	4+	2+	0	1+	0	0	0	0	0	0	0	0	0	0
M7053	4+	1+	4+	3+	0	2+	0	3+	0	0	0	0	0	0
M7060	4+	1+	4+	4+	0	3+	1+	2+	2+	2+	0	2+	1+	0
M7065	0	0	0	0	0	2+	0	0	0	0	0	0	0	0
M7100	0	0	0	0	0	tr	0	0	0	0	0	0	0	0
M7110	4+	2+	3+	4+	1+	0	0	2+	0	0	0	0	0	0
M7120	4+	3+	3+	3+	0	3+	0	2+	0	0	0	1+	0	0
M7126	3+	0	0	4+	0	0	0	0	0	3+	0	0	0	2+
M7127	4+	tr	0	3+	0	1+	0	0	0	1+	0	0	0	0
M7128	2+	0	0	0	0	0	0	0	0	2+	0	0	0	2+
M7129	0	0	0	0	0	0								
M7135	0	0	0	0	0	0								
M7137	2+	2+	0	0	0	0	0	0	0	0	0	0	0	0
M7152	0	0	2+	0	0	0	0	0	0	0	0	0	0	0
M7153	3+	3+	2+	3+	2+	1+	0	3+	0	0	0	2+	0	0
M7154	2+	0	2+	0	0	0	3+	2+	4+	3+	0	0	1+	0
M7155	2+	1+	0	2+	0	0	0	0	0	0	0	0	0	0
M7156	4+	0	0	3+	0	0	0	0	0	2+	0	0	0	2+
M7157	3+	0	0	2+	0	0	0	0	0	0	0	0	0	0

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M7158	1+	0	0	1+	0	0	0	0	0	2+	0	0	0	0
M7161	0	0	0	0	0	0								
M7162	3+	2+	0	3+	tr	0	0	0	0	3+	0	0	0	3+
M7165	3+	2+	0	3+	2+	0	0	0	0	0	0	2+	0	0
M7169	2+	0	1+	0	0	0	0	0	0	2+	0	0	0	2+
M7171	3+	3+	2+	2+	2+	1+	0	2+	0	0	0	2+	0	0
M7172	4+	3+	2+	3+	3+	2+	0	2+	0	0	0	1+	0	0
M7175	1+	1+	2+	0	0	0	0	0	1+	0	0	0	0	0
M7176	3+	1+	0	4+	0	0	0	0	0	0	0	0	0	0
M7177	3+	3+	2+	3+	3+	0	0	2+	2+	0	0	2+	1+	0
M7178	3+	3+	2+	2+	2+	1+	0	0	0	0	0	0	0	0
M7185	3+	0	0	2+	0	0	0	3+	0	0	0	0	0	0
M7186	tr	0	0	0	0	0	0	0	0	2+	0	0	0	2+
M7188	0	0	0	0	0	0								
M7197	3+	0	0	1+	0	0	0	0	0	0	0	0	0	0
M7199	0	0	0	0	0	0								
M7201	3+	0	0	2+	0	0	0	1+	0	0	0	0	0	0
M7202	4+	2+	1+	4+	2+	0	0	2+	0	3+	0	0	0	3+
M7204	3+	1+	1+	2+	0	1+	0	2+	0	0	0	1+	0	0
M7207	0	1+	1+	0	0	0	2+	1+	2+	3+	0	0	0	0
M7215	2+	0	0	1+	0	0	0	0	0	1+	0	0	0	0
M7216	0	0	0	0	0	0								
M7218	3+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M7219	3+	0	0	4+	0	0	0	0	0	0	0	0	0	0
M7223	2+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M7233	2+	0	0	1+	0	0	0	0	0	0	0	0	0	0
M7234	3+	2+	tr	2+	0	0	0	0	0	0	0	0	0	0
M7237	2+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M7238	0	0	0	0	0	0								

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M7258	0	0	0	0	0	0								
M7269	3+	1+	0	1+	0	0	0	0	0	0	0	0	0	0
M7273	2+	1+	1+	4+	tr	1+	0	2+	0	0	0	0	0	0
M7328	0	0	0	0	0	3+	0	0	0	2+	0	0	0	1+
M7334	0	0	0	0	0	0								
M7340	0	0	0	0	0	0								
M7350	3+	0	0	0	0	0	0	0	0	0	0	0	0	0
M7353	0	0	2+	1+	0	1+	2+	2+	2+	3+	1+	3+	3+	3+
M7361	4+	2+	3+	3+	0	0	0	3+	0	0	0	2+	2+	2+
M7365	0	0	0	0	0	0								
M7369	1+	0	0	2+	0	2+	2+	2+	0	3+	0	1+	2+	3+
M7380	3+	tr	1+	0	0	0	0	0	0	0	0	0	0	0
M7381	0	0	0	0	0	0								
M7382	0	0	0	0	0	0								
M7383	3+	2+	2+	2+	2+	2+	0	3+	0	0	0	3+	0	0
M7384	0	1+	0	0	0	0	0	tr	0	0	0	0	0	2+
M7385	3+	0	0	2+	0	1+	0	0	0	2+	0	0	0	1+
M7398	4+	2+	4+	1+	0	3+	0	2+	0	0	0	2+	0	0
M7399	4+	0	3+	2+	0	1+	0	2+	0	0	0	0	0	0
M7400	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M7401	4+	2+	2+	3+	0	2+	0	3+	2+	2+	0	1+	0	2+
M7406	0	0	0	0	0	0								
M7408	2+	0	0	3+	0	1+	0	0	0	2+	0	0	0	2+
M7409	1+	2+	2+	0	1+	2+	0	3+	0	2+	0	2+	0	0
M7411	0	0	0	1+	0	0	0	0	0	0	0	0	0	0
M7412	0	0	0	0	0	0								
M7416	2+	0	0	2+	0	2+	0	0	0	0	0	0	0	0
M7417	4+	0	2+	3+	0	0	0	0	0	0	0	2+	0	1+
M7418	4+	0	0	3+	0	0	0	2+	1+	0	0	0	0	0

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M7420	0	0	0	3+	0	1+	2+	2+	0	2+	0	2+	2+	2+
M7421	4+	0	0	3+	0	0	0	0	0	0	0	0	0	0
M7422	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
M7423	0	0	0	1+	0	1+	0	0	0	1+	0	0	0	1+
M7424	0	0	0	0	0	0								
M7425	4+	1+	1+	2+	0	0	0	3+	0	0	0	0	0	0
M7426	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
M7428	0	0	0	3+	0	1+	0	0	0	2+	0	1+	0	0
M7429	3+	0	1+	0	0	0	0	0	0	0	0	0	0	0
M7430	0	0	0	0	0	0								
M7431	0	0	0	0	0	0								
M7432	2+	0	0	2+	0	2+	0	3+	0	0	0	0	0	1+
M7433	4+	2+	3+	2+	0	2+	0	3+	0	0	0	2+	0	1+
M7434	4+	1+	3+	3+	0	1+	0	2+	0	0	0	0	0	2+
M7450	4+	0	0	4+	0	0	0	0	0	0	0	0	0	0
M7469	3+	0	0	0	0	2+	0	0	0	0	0	0	0	0
M7470	2+	0	0	0	0	2+	0	0	0	0	0	2+	2+	2+
M7474	0	0	2+	0	0	0	0	0	0	0	0	0	0	0
M7526	0	0	0	0	0	0								
M7528	0	2+	1+	0	1+	0	0	1+	0	0	0	0	0	0
M7532	0	0	0	0	0	0								
M7533	0	0	0	0	0	0								
M7534	0	0	0	0	0	0								
M7535	0	0	0	0	0	0								
M7536	0	0	0	0	0	0								
M7538	4+	2+	3+	3+	0	2+	0	3+	1+	0	0	2+	1+	0
M7633	0	0	0	0	0	0								
M7657	0	0	0	1+	0	0	0	0	0	0	0	0	0	0
M7673	4+	2+	2+	3+	2+	2+	0	1+	0	0	0	0	0	0

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M7674	2+	0	0	0	0	0	0	0	0	2+	0	0	0	0
M7675	3+	3+	3+	4+	3+	3+	0	3+	0	0	0	3+	0	1+
M7676	3+	3+	2+	3+	3+	2+	0	3+	0	1+	0	2+	0	3+
M7677	3+	3+	2+	3+	0	0	0	2+	0	3+	0	0	0	2+
M7678	2+	2+	0	3+	2+	1+	0	2+	0	0	0	1+	0	1+
M7679	0	0	0	0	0	0								
M7680	3+	0	0	4+	0	0	0	0	0	3+	0	0	0	3+
M7681	2+	3+	2+	3+	2+	2+	0	2+	0	1+	0	2+	0	2+
M7682	0	0	0	0	0	0								
M7684	0	0	0	0	0	0								
M7685	0	0	0	0	0	0								
M7686	0	0	0	0	0	0								
M7687	4+	3+	3+	3+	2+	2+	0	3+	0	0	0	2+	0	1+
M7688	4+	tr	0	4+	0	0	0	2+	0	3+	0	0	0	3+
M7759	3+	2+	2+	2+	2+	0	0	3+	0	2+	0	2+	2+	2+
M7844	0	0	0	0	0	1+	0	0	0	0	0	0	0	0
M7857	0	0	0	0	0	0								
M7877	0	0	0	0	0	0								
M7924	0	0	0	0	0	0								
M7928	0	0	0	0	0	0								
M7937	0	0	0	0	0	0								
M7943	0	0	0	0	0	0								
M7945	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M7947	0	0	0	0	0	0								
M8010	0	0	0	2+	0	0	0	0	0	0	0	0	0	0
M8144	4+	3+	1+	2+	2+	0	0	2+	0	0	0	0	0	0
M8158	0	0	0	0	0	0								
M8159	3+	2+	2+	1+	1+	2+	0	3+	0	2+	0	2+	2+	3+
M8160	3+	2+	0	2+	0	0	0	0	0	0	0	0	0	0

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M8161	3+	2+	1+	2+	1+	0	0	2+	1+	2+	0	1+	2+	2+
M8162	4+	2+	2+	3+	0	0	0	2+	0	2+	0	0	0	1+
M8163	0	0	0	0	0	0								
M8215	3+	0	0	2+	0	0	0	1+	0	3+	0	1+	0	0
M8393	3+	0	0	1+	0	0	0	0	2+	3+	0	0	0	3+
M8395	0	0	0	0	0	0								
M8396	0	0	0	0	0	0								
M8409	0	0	0	0	0	0								
M8430	0	0	0	0	0	0								
M8461	0	0	0	0	0	0								
M8462	2+	0	0	0	0	0	0	0	0	2+	0	0	0	0
M8466	0	0	0	0	0	0								
M8468	tr	0	0	0	0	0	0	0	0	0	0	0	0	0
M8471	0	0	0	0	0	0								
M8472	3+	0	0	3+	0	0	0	0	0	0	0	0	0	0
M8473	2+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M8474	0	0	0	0	0	0								
M8475	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8477	0	0	0	0	0	0								
M8478	3+	0	0	3+	0	0	0	0	0	4+	0	0	0	4+
M8480	4+	3+	1+	2+	0	0	0	3+	0	1+	0	0	0	0
M8481	2+	2+	1+	tr	tr	0	0	3+	0	2+	0	0	0	0
M8483	0	0	0	0	0	0								
M8485	3+	0	0	tr	0	0	0	0	0	0	0	0	0	0
M8486	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8487	0	0	0	0	0	0								
M8488	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8489	0	0	0	0	1+	0	0	0	0	0	0	2+	0	0
M8490	2+	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M8491	0	0	0	0	0	0								
M8493	0	0	0	0	0	0								
M8494	0	0	0	0	0	0								
M8495	3+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M8496	2+	0	0	2+	0	0	0	0	0	2+	0	0	0	1+
M8497	4+	3+	1+	3+	2+	0	0	2+	0	0	0	0	0	0
M8498	4+	3+	1+	1+	2+	0	0	3+	0	0	0	0	0	0
M8499	0	0	0	0	0	0								
M8500	0	0	0	0	0	0								
M8502	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8503	0	0	0	0	0	0								
M8505	2+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M8507	0	0	0	0	0	0								
M8508	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8510	3+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8511	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8512	2+	3+	3+	2+	2+	2+	0	1+	0	0	0	2+	0	0
M8513	tr	0	0	0	0	0	0	0	0	0	0	0	0	0
M8514	3+	0	0	1+	2+	1+	0	0	0	0	0	2+	0	0
M8515	4+	3+	3+	3+	3+	2+	0	3+	0	0	0	3+	0	0
M8516	2+	2+	1+	0	1+	0	0	0	0	0	0	0	0	0
M8517	2+	0	0	2+	0	0	0	0	0	2+	0	0	0	0
M8518	0	0	0	0	0	0								
M8519	0	0	0	0	0	0								
M8520	4+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M8521	0	0	0	0	0	0								
M8522	2+	1+	0	1+	0	0	0	2+	0	0	0	0	0	0
M8523	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8526	0	0	0	0	0	0								

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M8557	2+	0	1+	2+	0	0	0	2+	1+	1+	0	1+	0	2+
M8558	3+	0	0	2+	0	0	0	2+	1+	1+	0	0	0	1+
M8559	2+	0	0	2+	0	0	0	0	0	0	0	1+	0	0
M8560	2+	0	1+	0	0	0	0	1+	0	0	0	0	0	0
M8561	0	0	0	0	0	0								
M8562	0	0	2+	0	0	0	0	3+	0	0	0	2+	0	0
M8563	1+	0	0	1+	0	0	0	2+	0	0	0	2+	0	0
M8564	2+	0	0	1+	0	0	0	1+	0	1+	0	0	0	0
M8598	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8687	2+	0	0	0	0	0	0	2+	0	0	0	0	0	0
M8704	0	0	0	0	0	0								
M8731	3+	0	1+	1+	0	0	0	0	0	0	0	0	0	2+
M8760	2+	0	0	1+	0	0	0	0	0	0	0	0	0	0
M8774	1+	0	0	2+	0	0	0	0	0	0	0	0	0	0
M8802	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8824	2+	0	tr	3+	0	1+	0	0	0	2+	0	1+	0	2+
M8825	3+	tr	1+	2+	0	0	0	1+	0	3+	0	1+	1+	4+
M8826	3+	0	0	2+	0	2+	0	0	0	3+	0	2+	0	3+
M8827	3+	0	0	3+	0	3+	0	0	0	0	0	0	0	0
M8828	3+	2+	3+	1+	3+	1+	0	2+	0	0	0	2+	0	2+
M8829	0	0	0	2+	0	0	0	0	2+	0	0	0	0	2+
M8851	0	0	0	0	0	0								
M8852	1+	0	0	0	0	0	0	0	0	0	0	0	0	0
M8886	3+	1+	1+	3+	2+	0	2+	3+	1+	3+	2+	3+	3+	H
M8887	2+	0	2+	1+	0	0	0	0	0	0	0	0	0	0
M8894	0	0	0	0	0	0								
M8950	0	0	0	0	0	0								
M8959	3+	2+	3+	3+	1+	1+	0	3+	0	0	0	2+	0	0
M8985	4+	0	0	3+	0	0	0	0	0	2+	0	0	0	3+

Table 2 (continued)

	Detection Screen						Identification Screen							
	COLD			WARM			COLD				WARM			
Sample ID	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1,3,4,5,7	1.3,4,w7	1.2,4,7	1.1	1.1,4,5,7	negative	3,4,5	1.1	1.1,4,5,7	negative	3,4,5
M8986	0	0	1+	0	0	0	0	0	0	0	0	0	0	0
M8987	3+	0	1+	2+	0	0	0	3+	0	0	0	1+	0	1+
M9001	1+	0	0	0	0	0	0	0	2+	3+	0	0	0	3+
M9002	0	0	0	0	0	0								
M9003	2+	0	1+	2+	0	0	0	2+	2+	3+	0	2+	0	2+
M9004	3+	0	1+	2+	0	2+	0	3+	0	2+	0	3+	0	3+
M9005	0	0	0	0	0	0								
M9006	0	2+	0	0	0	0	0	0	0	2+	0	0	0	2+
M9007	1+	0	0	0	0	0	0	2+	0	3+	0	1+	0	3+
M9008	3+	3+	2+	3+	2+	2+	3+	3+	4+	4+	3+	3+	3+	4+
M9009	3+	0	2+	3+	0	0	0	3+	0	3+	0	2+	0	2+
M9014	0	0	0	0	0	0								
M9015	2+	0	1+	0	0	0	0	0	3+	3+	1+	1+	2+	3+
M9099	0	0	0	1+	0	0	0	0	0	0	0	0	0	0
M9101	2+	0	0	tr	0	0	0	2+	0	0	0	0	0	2+
M9111	2+	0	0	0	0	0	0	2+	0	0	0	0	0	0
M9112	3+	0	0	3+	0	0	0	1+	0	0	0	1+	0	0
M9113	2+	0	0	1+	0	0	0	2+	0	0	0	1+	0	2+
M9114	1+	0	0	1+	0	0	0	0	0	0	0	0	0	0
M9127	2+	0	0	0	0	0	0	2+	0	2+	0	3+	2+	3+
M9128	3+	0	0	2+	0	0	0	0	0	3+	0	0	0	3+
M9205	0	0	0	0	0	0								
M9215	2+	0	0	1+	0	0	0	4+	0	3+	0	2+	0	2+
M9219	3+	0	1+	0	0	0	0	1+	3+	2+	0	0	0	2+
M9221	0	1+	0	0	0	0	0	0	2+	2+	tr	1+	1+	3+
M9226	3+	0	1+	2+	0	0	0	3+	0	3+	0	1+	2+	3+
Zorro	3+	0	0	1+	0	1+	0	2+	0	3+	0	2+	0	2+

H = complete hemolysis of red blood cells

tr = trace reaction

The reaction data were evaluated within these groups to detect patterns of reactivity. Within each group, the reactions were divided into categories of samples reacting with one, two, or three dogs, and were further evaluated based on the antigenic profile of the dog or dogs reacting.

A total of 88 samples reacted with only one dog in the Detection Screen (Table 4). Seventy-seven samples reacted with the DEA 1.1,3,4,5,7 positive dog. Seven samples reacted with the DEA 1.2,4,7 positive dog and four samples reacted with the DEA 1.3,4,w7 positive dog.

Table 4: Samples reacting with only one dog in the Detection Screen

DEA Pattern	Cold	Warm	Cold & Warm	Total
1.1,3,4,5,7	29	5	43	77
1.2,4,7	4	3	0	7
1.3,4,w7	3	1	0	4
Total	36	9	43	88

Forty-eight samples reacted with a two dog pair in the Detection Screen (Table 5). Thirty-six samples reacted with the DEA 1.1,3,4,5,7 positive and DEA 1.2,4,7 positive dog pair. Ten samples reacted with the DEA 1.1,3,4,5,7 positive and DEA 1.3,4,w7 positive dog pair. Only 2 samples reacted with the DEA 1.2,4,7 positive and DEA 1.3,4,w7 positive dog pair.

Table 5: Samples reacting with a two dog pair in the Detection Screen

DEA Pattern	Cold Only	Warm Only	Cold & Warm	Total
1.1,3,4,5,7 & 1.2,4,7	7	3	26	36
1.1,3,4,5,7 & 1.3,4,w7	1	0	9	10
1.2,4,7 & 1.3,4,w7	1	0	1	2
Total	9	3	36	48

Forty-eight serum samples reacted with all three dogs in the Detection Screen (Table 6). Only two of the 48 samples reacted only at cold conditions. The other 46 samples reacted at both cold and warm conditions.

Table 6: Samples reacting with all three dogs in the Detection Screen

DEA Pattern	Cold Only	Warm Only	Cold & Warm	Total
1.1,3,4,5,7; 1.2,4,7 & 1.3,4,w7	2	0	46	48

Identification Screen

A total of 184 serum samples were rescreened to identify the anti-DEA antibody detected in the Detection Screen (Table 7). Identification Screening was conducted using dogs with the antigenic profiles of DEA 1.1 positive, DEA 1.1,4,5,7 positive, DEA negative, and DEA 3,4,5 positive, as previously described. This screening resulted in 62 negative reactions (33.7%). Of the remaining 122 samples, a total of 84 samples (45.7%) showed positive reactions at both RT/4°C (cold) and 37°C/ACG (warm) conditions, 30 samples (16.3%) were positive only at cold conditions, and 8 samples (4.3%) were positive only at warm conditions.

Table 7: Summary of reaction conditions in the Identification Screen

Reaction Pattern	Number of Samples	Percentage of Total (%)
Negative	62	33.7%
Positive cold & warm	84	45.7%
Positive cold, Negative warm	30	16.3%
Negative cold, Positive warm	8	4.3%
Total	184	100%

The reaction data were evaluated within these groups to detect patterns of reactivity. Within each group, the reactions were divided into categories of samples reacting with one, two, three, or four dogs, and were further evaluated based on the antigenic profile of the dog or dogs reacting. Dividing the Identification Screen data alone into categories based on these criteria did not provide useful patterns.

Combined Results

The results from the Detection Screen were combined with the results from the Identification Screen to give complete screening results. A total of 81 samples (30.6%) were completely negative (Table 8). Of the remaining 184 samples, 143 samples (54.0%) reacted at both warm and cold conditions. Thirty-four samples (12.8%) reacted only at cold conditions, and 7 samples (2.6%) reacted only at warm conditions.

Table 8: Summary of reaction conditions for combined results of Detection and Identification Screens

Reaction Pattern	Number of Samples	Percentage of Total (%)
Negative	81	30.6%
Positive cold & warm	143	54.0%
Positive cold, Negative warm	34	12.8%
Positive warm, Negative cold	7	2.6%
Total	265	100%

The combined positive reaction patterns of the Detection and Identification screens are summarized in Table 9.

Table 9: Reaction patterns of screening dogs as related to individual serum antibody specificities for combined results of the Detection and Identification Screens

Dogs Reacting							Number of Samples Demonstrating Pattern	Distribution of Samples per Reaction Condition	Antibody Specificity
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA Negative	DEA 3,4,5			
+	0	0	+	0	0	0	0	0	
0	+	0	+	0	0	0	0	0	
0	0	+	+	0	0	0	0	0	
+	+	0	+	0	0	0	0	0	
+	0	+	+	0	0	0	0	0	
0	+	+	+	0	0	0	0	0	
+	+	+	+	0	0	0	0	0	
+	0	0	0	+	0	0	8	3 Cold, 5 Both	
0	+	0	0	+	0	0	1	1 Both	
0	0	+	0	+	0	0	1	1 Warm	
+	+	0	0	+	0	0	3	1 Cold, 2 Both	anti-DEA 7*
+	0	+	0	+	0	0	2	2 Both	
0	+	+	0	+	0	0	1	1 Both	
+	+	+	0	+	0	0	20	20 Both	anti-DEA 7*
+	0	0	0	0	+	0	0	0	
0	+	0	0	0	+	0	0	0	
0	0	+	0	0	+	0	0	0	
+	+	0	0	0	+	0	0	0	
+	0	+	0	0	+	0	0	0	
0	+	+	0	0	+	0	0	0	
+	+	+	0	0	+	0	1	1 Cold	
+	0	0	0	0	0	+	14	2 Cold, 12 Both	anti-DEA 3
0	+	0	0	0	0	+	1	1 Both	
0	0	+	0	0	0	+	2	2 Both	

Table 9 (continued)

Dogs Reacting							Number of Samples Demonstrating Pattern	Distribution of Samples per Reaction Condition	Antibody Specificity
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA Negative	DEA 3,4,5			
+	+	0	0	0	0	+	6	6 Both	
+	0	+	0	0	0	+	1	1 Both	
0	+	+	0	0	0	+	0	0	
+	+	+	0	0	0	+	1	1 Both	
+	0	0	+	+	0	0	0	0	anti-DEA 1.1
0	+	0	+	+	0	0	0	0	
0	0	+	+	+	0	0	0	0	
+	+	0	+	+	0	0	0	0	
+	0	+	+	+	0	0	0	0	
0	+	+	+	+	0	0	0	0	
+	+	+	+	+	0	0	0	0	
+	0	0	+	0	+	0	0	0	
0	+	0	+	0	+	0	0	0	
0	0	+	+	0	+	0	0	0	
+	+	0	+	0	+	0	0	0	
+	0	+	+	0	+	0	0	0	
0	+	+	+	0	+	0	0	0	
+	+	+	+	0	+	0	0	0	
+	0	0	+	0	0	+	0	0	
0	+	0	+	0	0	+	0	0	
0	0	+	+	0	0	+	0	0	
+	0	+	+	0	0	+	0	0	
0	+	+	+	0	0	+	0	0	
+	+	+	+	0	0	+	0	0	
+	0	0	0	+	+	0	1	1 Both	

Table 9 (continued)

Dogs Reacting							Number of Samples Demonstrating Pattern	Distribution of Samples per Reaction Condition	Antibody Specificity
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA Negative	DEA 3,4,5			
+	+	0	+	0	0	+	0	0	
0	+	0	0	+	+	0	0	0	
0	0	+	0	+	+	0	0	0	
+	+	0	0	+	+	0	0	0	
+	0	+	0	+	+	0	0	0	
0	+	+	0	+	+	0	0	0	
+	+	+	0	+	+	0	2	2 Both	
+	0	0	0	+	0	+	8	8 Both	anti-DEA 5
0	+	0	0	+	0	+	0	0	
0	0	+	0	+	0	+	0	0	
+	+	0	0	+	0	+	9	9 Both	
+	0	+	0	+	0	+	0	0	
0	+	+	0	+	0	+	0	0	
+	+	+	0	+	0	+	14	1 Cold, 13 Both	anti-DEA 4
+	0	0	0	0	+	+	3	3 Both	
0	+	0	0	0	+	+	0	0	
0	0	+	0	0	+	+	0	0	
+	+	0	0	0	+	+	0	0	
+	0	+	0	0	+	+	0	0	
0	+	+	0	0	+	+	0	0	
+	+	+	0	0	+	+	0	0	
0	+	0	+	+	+	0	0	0	
0	0	+	+	+	+	0	0	0	
+	0	0	+	+	+	0	0	0	
+	+	0	+	+	+	0	0	0	

Table 9 (continued)

Dogs Reacting							Number of Samples Demonstrating Pattern	Distribution of Samples per Reaction Condition	Antibody Specificity
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA Negative	DEA 3,4,5			
+	0	+	+	+	+	0	0	0	
0	+	+	+	+	+	0	0	0	
+	+	+	+	+	+	0	0	0	
+	0	0	+	+	0	+	0	0	
0	+	0	+	+	0	+	0	0	
0	0	+	+	+	0	+	0	0	
+	+	0	+	+	0	+	0	0	
+	0	+	+	+	0	+	0	0	
0	+	+	+	+	0	+	0	0	
+	+	+	+	+	0	+	0	0	
+	0	0	+	0	+	+	0	0	
0	+	0	+	0	+	+	0	0	
0	0	+	+	0	+	+	0	0	
+	+	0	+	0	+	+	0	0	
+	0	+	+	0	+	+	0	0	
0	+	+	+	0	+	+	0	0	
+	+	+	+	0	+	+	0	0	
+	0	0	0	+	+	+	2	2 Both	
0	+	0	0	+	+	+	0	0	
0	0	+	0	+	+	+	1	1 Cold	
+	+	0	0	+	+	+	6	6 Both	
+	0	+	0	+	+	+	0	0	
+	+	+	0	+	+	+	5	5 Both	
+	0	0	+	+	+	+	0	0	
0	+	0	+	+	+	+	0	0	
0	+	+	0	+	+	+	0	0	

Table 9 (continued)

Dogs Reacting							Number of Samples Demonstrating Pattern	Distribution of Samples per Reaction Condition	Antibody Specificity
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA Negative	DEA 3,4,5			
0	0	+	+	+	+	+	0	0	
+	+	0	+	+	+	+	5	5 Both	
+	0	+	+	+	+	+	0	0	
0	+	+	+	+	+	+	1	1 Cold	
+	+	+	+	+	+	+	3	3 Both	
+	0	0	0	0	0	0	41	18 Cold, 4 Warm, 19 Both	
0	+	0	0	0	0	0	5	3 Cold, 2 Warm	anti-DEA 1.2
0	0	+	0	0	0	0	0	0	anti-DEA 1.3
+	+	0	0	0	0	0	7	2 Cold, 5 Both	
+	0	+	0	0	0	0	7	1 Cold, 6 Both	
0	+	+	0	0	0	0	0	0	
+	+	+	0	0	0	0	2	2 Both	

*There are two possible patterns considered for anti-DEA 7 due to the inclusion of a dog with weakly expressed DEA 7. The weak expression of DEA 7 may lead to a negative reaction in the presence of a low titer of anti-DEA 7.

Cold = Samples reacted at immediate spin, room temperature, and/or 4°C only.
Warm = Samples reacted at 37°C, and/or ACG only.
Both = Samples reacted at cold conditions and warm conditions.

Controls

The positive and negative control pair was run on each day of screening during both the Detection and Identification Screens. The control pair was run a total of 40 times, with only 1 failure of the negative control on Day 1 of this study due to the use of an improper reagent. The data collected from Day 1 were excluded from the results due to the control failure. The positive controls did not fail during the study, consistently giving reactions of a 3+ or 4+ strength.

The check cells were run with each sample that was negative after the ACG phase. The only check cell failure occurred on Day 3 of testing due to a technical error in the preparation of the reagent. The data collected from that day were excluded from the results.

DISCUSSION

A total of 265 dog serum samples were screened for anti-DEA activity and 184 of those samples demonstrated reactivity. Those 184 samples were rescreened with a second group of dog red blood cells to identify the specificity of the reacting anti-DEA.

During the Detection Screen, a total of 81 samples (30.6%) were negative for anti-DEA activity. Seventy-seven samples (29.1%) reacted with the DEA 1.1,3,4,5,7 positive dog only and were suspected of containing anti-DEA 1.1, anti-DEA 3, or anti-DEA 5 because this dog was the only Detection Screen dog to express DEA 1.1, 3, and 5 (Table 10). Seven samples (2.6%) reacted only with the DEA 1.2,4,7 positive dog and were suspected of containing anti-DEA 1.2 because the only known DEA that was unique to this dog was DEA 1.2. Only 4 samples (1.5%) reacted with the DEA 1.3,4,7 positive dog alone and were suspected of containing anti-DEA 1.3 because this dog was the only Detection Screen dog to express DEA 1.3. Thirty-six samples (13.6%) reacted with both the DEA 1.1,3,4,5,7 positive and DEA 1.2,4,7 positive dogs but not the DEA 1.3,4,w7 dog, and were suspected of containing anti-DEA 7 because both dogs expressed DEA 7. The samples were not suspected of containing anti-DEA 4 because they did not react with the DEA 1.3,4,w7 dog. If anti-DEA 4 had been present, the sample would be expected to react with all three dogs. The other Detection Screen dog, DEA 1.3,4,w7 positive, also expresses DEA 7, but not as strongly as most DEA 7 positive dogs, and may result in false negative reactions.

Table 10: Reaction patterns of screening dogs as related to individual serum antibody specificities during Detection Screening

Dogs Reacting			Antibody Specificity	Number of Samples Demonstrating Pattern	Percent (%) of Samples
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7			
+	0	0	anti-DEA 1.1, anti-DEA 3, or anti-DEA 5	77	29.1%
0	+	0	anti-DEA 1.2	7	2.6%
0	0	+	anti-DEA 1.3	4	1.5%
+	+	0	anti-DEA 7*	36	13.6%
+	0	+	unknown	10	3.8%
0	+	+	unknown	2	0.7%
+	+	+	anti-DEA 4, or anti-DEA 7*	48	18.1%
0	0	0	negative	81	30.6%
			TOTAL	265	100%

*There are two possible patterns considered for anti-DEA 7 due to the inclusion of a dog with weakly expressed DEA 7. The weak expression of DEA 7 may lead to a negative reaction in the presence of a low titer of anti-DEA 7.

As a result, anti-DEA 7 may be detected by the DEA 1.1,3,4,5,7 positive and DEA 1.2,4,7 positive pair or with all three Detection Screen dogs. Forty-eight samples (16.6%) reacted with all three Detection Screen dogs and were suspected of anti-DEA 4 or anti-DEA 7 because the common antigens among the three dogs were DEA 4 and 7. A total of 12 samples (4.4%) reacted with either the DEA 1.1,3,4,5,7 positive and DEA1.3,4,w7 positive pair or the DEA 1.2,4,7 positive and DEA 1.3,4,w7 positive pair, and were suspected of containing an unknown antibody because there were no known DEAs that were common to the dogs in each pair. An unknown antibody, a false positive, or a false negative for a different reaction pattern may be a consideration in each of the reaction patterns previously mentioned.

Data were evaluated for the Identification Screen alone and no patterns of significance were detected. However, sixty-two samples (33.7%) failed to react with any of the Identification Screen dogs. The Identification Screen panel of dogs did not represent DEA 1.2 or DEA 1.3, suggesting that the non-reactive samples may contain anti-DEA 1.2 or anti-DEA 1.3. Naturally-occurring antibodies against DEA 1.2 and DEA 1.3 have not been previously reported, however, these antigens have been reported to induce antibody production in dogs negative for the corresponding antigen.[33] Since the DEA exposure histories of the dogs for which serum samples were submitted are unknown, anti-DEA 1.2 and anti-DEA 1.3 may be possibilities. It must be noted, however, that these two antigens have p-values greater than 0.05, and therefore lie outside the 95% confidence interval.

Combined results from the Detection Screen and Identification Screen show a total of 81 samples (30.6%) as being negative for anti-DEA activity, which is based on the results from the Detection Screen. None of the samples reacted with the DEA 1.1,3,4,5,7 positive, DEA 1.1 positive, and DEA 1.1,4,5,7 positive dog group, suggesting that there were no anti-DEA 1.1 antibodies present in the samples as these dogs are the only DEA 1.1 positive dogs in the study (Table 9, Table 11). Anti-DEA 1.1 has not been reported to be a naturally-occurring

Table 11: Prevalence of Anti-DEA Antibodies

Antibody	Prevalence (%)	Previously Described
Anti-DEA 1.1	0%	no
Anti-DEA 1.2	1.9%	no
Anti-DEA 1.3	0%	no
Anti-DEA 3	5.3%	yes
Anti-DEA 4	unlikely	no
Anti-DEA 5	3%	yes
Anti-DEA 7	8.7%	yes
Multiple Antibodies	*	*
Unknown Antibodies	Up to 45.3%	no

* An accurate prevalence of multiple antibody combinations could not be determined. Previous studies have not described the prevalence of multiple antibody combinations.

antibody, but it can be induced upon exposure of a DEA 1.1-negative dog to DEA 1.1. Five samples (1.9%) reacted with the DEA 1.2,4,7 positive dog alone and are suspected of containing anti-DEA 1.2. Anti-DEA 1.2 has not been reported to occur naturally, but agglutinating antibody can be induced in a DEA 1.2-negative dog after exposure to DEA 1.2.[24,34] The serum samples are from dogs of unknown exposure history, so it is possible that an acquired anti-DEA 1.2 is present in this population. The red blood cell panel did not meet the required p-

value of 0.05 or less for DEA 1.2 needed for proper antibody determination, so there is a greater than 1 in 20 chance that the pattern for anti-DEA 1.2 may be another specificity.[11] No samples reacted with the DEA 1.3,4,w7 positive dog alone, suggesting that there were no anti-DEA 1.3 antibodies present in the samples. Anti-DEA 1.3 has not been indicated as a naturally-occurring antibody. Antibody formation has been documented in a DEA 1.3-negative dog following exposure to DEA 1.3, although the antibody described was reactive with DEA 1.1 and DEA 1.2.[33] Fourteen samples (5.3%) reacted with the DEA 1.1,3,4,5,7 positive and DEA 3,4,5 positive dog pair and are suspected of containing anti-DEA 3 or an unknown antibody. This supports previous studies in which anti-DEA 3 has been reported to occur naturally.[12,15] Fourteen samples (5.3%) reacted with the DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, DEA 1.3,4,w7 positive, DEA 1.1,4,5,7 positive, and DEA 3,4,5 positive dog group and are suspected of containing anti-DEA 4, an unknown antibody, or a combination of antibodies. Anti-DEA 4 has not been reported as a naturally-occurring antibody and is not likely to be seen frequently in the population since 98% of dogs express DEA 4. Eight samples (3%) reacted with the DEA 1.1,3,4,5,7 positive, DEA 1.1,4,5,7 positive, and DEA 3,4,5 positive dog group and are suspected of containing anti-DEA 5 since they are the only samples that contain DEA 5. Anti-DEA 5 has previously been reported as a naturally-occurring antibody. Two groupings of reaction patterns were suspected of containing anti-DEA 7 due to the weak expression of DEA 7 in the DEA 1.3,4,w7 positive dog – the DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, and DEA 1.1,4,5,7 positive dog group

and the DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, DEA 1.3,4,w7 positive, and DEA 1.1,4,5,7 positive dog group. The DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, and DEA 1.1,4,5,7 positive dog group reacted with 3 samples (1.1%) and the DEA 1.1,3,4,5,7 positive, DEA 1.2,4,7 positive, DEA 1.3,4,w7 positive, and DEA 1.1,4,5,7 positive dog group reacted with 20 samples (7.5%), giving a total of 23 samples (8.7%) that are suspected of containing anti-DEA 7. Anti-DEA 7 has been reported to be naturally-occurring in some DEA 7-negative dogs. A total of 120 samples (45.3%) reacted but did not show activity specific to any known individual anti-DEA antibody. Any of the reaction patterns may also represent antibodies to unknown DEAs.

Sixty-two samples (23%) that reacted during the Detection Screen did not demonstrate reactions in the Identification Screen. Of these 62 samples, 24 samples (38.7%) were only reactive at cold conditions, indicating a potential cold agglutinin. Six samples (9.7%) were only reactive at warm conditions, and 32 samples (51.6%) were reactive for both cold and warm conditions. Of the 62 samples, 41 samples (66.1%) were reactive with the DEA 1.1,3,4,5,7 positive dog only, which suggests the dog may express an unknown DEA. Five samples (8%) reacted with the DEA 1.2,4,7 positive dog only, suggesting another potential unknown DEA.

When considering the presence of multiple anti-DEA antibodies, sixteen samples of the 120 samples with unknown specificities may be explained (Table

12). The multiple antibody combination of anti-DEA 1.2,3 or anti-DEA 1.3,3 explains 7 samples. The remaining 9 samples have numerous possible multiple antibody combinations: anti-DEA 3,7; anti-DEA 5,7; anti-DEA 3,5,7; anti-DEA 1.2,3,5; anti-DEA 1.2,3,7; anti-DEA 1.2,5,7; anti-DEA 1.2,3,5,7 (Table 12). Four patterns previously described for individual antibodies anti-DEA 4, anti-DEA 5, and anti-DEA 7 also correspond to multiple antibody patterns. The pattern for the individual anti-DEA 5 antibody may also represent the combination of anti-DEA 3,5. The two patterns for anti-DEA 7 may also correspond to anti-DEA 1.2,7 and anti-DEA 1.3,7. Although anti-DEA 4 is included in the possible specificities for both individual and multiple antibodies, it is not likely to be demonstrated frequently as an individual antibody as indicated in Table 11 due to the high prevalence of DEA 4 (98% positive). A more likely explanation for the pattern corresponding to the individual anti-DEA 4 specificity is the presence of multiple antibodies. There are numerous possible multiple antibody combinations corresponding to the pattern for anti-DEA 4: anti-DEA 3,7; anti-DEA 5,7; anti-DEA 3,5,7; anti-DEA 1.2,3,7; anti-DEA 1.2,5,7; anti-DEA 1.3,3,7; anti-DEA 1.3,5,7; anti-DEA 1.3,3,5,7; or any of these combinations including anti-DEA 4.

When the results of the Detection Screen and Identification Screen were combined, the percentage of samples that reacted at both cold and warm conditions increased, while the percentage of samples reacting only at cold conditions or only at warm conditions decreased (Table 13). This suggests that a portion of the samples that demonstrated reactions only at cold conditions or only

Table 12: Reaction patterns of screening dogs as related to multiple serum antibody specificities for combined results of the Detection and Identification Screens

Dogs Reacting							Multiple Antibody Specificity	Number of Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA Negative	DEA 3,4,5		
+	0	0	0	+	0	+	anti-3,5	8 *
+	+	+	0	+	0	+	anti-3,7 anti-5,7 anti-3,5,7 anti-1.2,3,7 anti-1.2,5,7 anti-1.2,3,5,7 anti-1.3,3,7 anti-1.3,5,7 anti-1.3,3,5,7 (and any of these combinations with anti-4)	14‡
+	+	0	0	+	0	+	anti-3,7 anti-5,7 anti-3,5,7 anti-1.2,5 anti-1.2,3,5 anti-1.2,3,7 anti-1.2,5,7 anti-1.2,3,5,7	9
+	0	0	+	+	0	+	anti-1.1,3,5	0
+	0	+	0	+	0	+	anti-1.3,3,5	0

Table 12 (continued)

Dogs Reacting							Multiple Antibody Specificity	Number of Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	**DEA 1.2,4,7**	**DEA 1.3,4,w7**	**DEA 1.1**	**DEA 1.1,4,5,7**	**DEA Negative**	**DEA 3,4,5**		
+	+	+/0	+	+	0	+	anti-1.1,3,7 anti-1.1,5,7 anti-1.1,3,5,7	0
+	0	0	+	+	0	+	anti-1.1,3 anti-1.1,5	0
+	+	+/0	+	+	0	0	anti-1.1,7	0
+	+	0	0	0	0	+	anti-1.2,3	6
+	+	0	0	+	0	0	anti-1.2,7	3§
+	+	+	0	+	0	0	anti-1.2,7 anti-1.3,7	20§
+	0	+	0	0	0	+	anti-1.3,3	1
+	0	+	0	+	0	+	anti-1.3,5	0
							TOTAL	51

* The reaction pattern associated with this multiple antibody combination is the same pattern specific for the individual anti-DEA 5 antibody.

‡ The reaction pattern associated with this multiple antibody combination is the same pattern specific for the individual anti-DEA 4 antibody.

§ The reaction pattern associated with this multiple antibody combination is the same pattern specific for the individual anti-DEA 7 antibody.

Table 13: Reaction patterns of screening dogs as related to temperature of reaction in the Detection Screen, Identification Screen, and for combined results

Reaction Pattern	Detection Screen	Identification Screen	Combined Results
Negative	30.6% (81/265)	33.7% (62/184)	30.6% (81/265)
Positive Cold, Positive Warm	47.2% (125/265)	45.7% (84/184)	54.0% (143/265)
Positive Cold, Negative Warm	17.7% (47/265)	16.3% (30/184)	12.8% (34/265)
Positive Warm, Negative Cold	4.5% (12/265)	4.3% (8/184)	2.6% (7/265)
Total	100% (265/265)	100% (184/184)	100% (265/265)

at warm conditions for one screening stage, either the Detection Screen or the Identification Screen, were capable of demonstrating reactions at the other condition as well and did so in the other screening stage. The variability in the conditions at which a sample will react may be important when considering whether the antibody is of significance, because an antibody that is defined as "cold-reacting" may still be capable of reacting at warm conditions and therefore be of concern in the animal.

The p-value was calculated for each clinically-significant DEA in the red blood cell panel to ensure that each DEA was sufficiently represented. A p-value of equal to or less than 0.05 indicated that there was equal to or less than a 1 in 20 chance that the antibody identified was of a different specificity than the one indicated by the reaction pattern. This ensures with 95% confidence that the antibody specificity assigned to a reaction pattern is the correct specificity. All DEAs with the exception of DEA 1.2 and DEA 1.3 had a p-value of equal to or less than 0.05 as indicated in Table 1. Both DEA 1.2 and DEA 1.3 had a p-value of 0.057 or 0.143 depending on the method used to calculate probability (Harris

and Hochman method or Fisher Exact method, respectively). These p-values indicate a less than 95% level of confidence that the antibody has been correctly identified, or greater than 1 in 20 likelihood that antibody was incorrectly identified.

Differing Definitions in Dog Immunohematology

There is debate within the dog immunohematology community as to the definition of a "positive" reaction and a "negative" reaction. A prevalent definition of "positive" is any reaction 2+ or greater, and the correlating definition of "negative" is any reaction 1+ or less. This study utilized a definition of "positive" as a 1+ reaction or greater and "negative" as a 0 or trace reaction, as was described in the Materials and Methods section of this document. This difference in the definition of what constitutes a "positive" or a "negative" may impact the reported prevalence of antibodies from previous studies. In this study, changing the definition of "positive" from 1+ and greater to 2+ and greater resulted in 22 additional negative samples for a total of 103 samples (38.9%). Of these 22 negative samples, 6 samples previously could be assigned a DEA specificity with the 1+ critieria. The change in criteria also affected the categorization of 60 more samples. Seventeen of the 60 samples changed from having a defined specificity to having an unknown specificity, while 5 samples that were previously uncategorized became categorized with a specificity. Thirteen samples that previously had a specificity were changed to a different specificity when using the 2+ criteria. Twenty-two more samples had an unknown specificity with the

original criteria and continued to have an unknown specificity with the 2+ criteria, although the reaction pattern was different. Due to the weak DEA 7 included in the antigenic panel, two different reaction patterns were indicative of an anti-DEA 7. With this consideration, three samples were defined as anti-DEA 7 or a multiple antibody combination containing anti-DEA 7 at either criteria, but had different reaction patterns for each.

Additional factors that may influence the reaction strength thereby impacting whether or not a reaction is considered positive are the variability in the titer of antibody, varying dosage of antigen on the red blood cell, and technique of the individual technician. These factors can influence the strength or perceived strength of the reaction, potentially leading to false negatives. A final consideration is the reaction patterns of 1+ samples in this study. Nine samples demonstrated 1+ reactions in the Detection Screen, but demonstrated 2+ or greater reactions in the Identification Screen, suggesting they were positive. One hundred seventeen samples (44.2%) demonstrated a 1+ reaction with at least one dog in the red blood cell panel for either the Detection or Identification Screens. Of these 117 samples, 82 samples (70.1%) had a different reaction pattern when the 1+ reaction was disregarded, as mentioned previously. Of these 82 samples, 39 samples (47.6%) demonstrated a defined antibody specificity when considering the 1+ reaction as valid. This, again, suggests the samples did contain an anti-DEA antibody or combination of anti-DEA antibodies. Additionally, the Negative Control did not demonstrate a 1+ reaction at any time during this study. All results for the Negative Control

indicated a reaction strength of 0, showing that a negative reaction should react at a strength of 0.

Possible Explanations for Unknown or Inconsistent Results

Samples that did not react in patterns consistent with individual or multiple known antibodies may contain antibodies to other DEAs. Previous studies have documented the presence of other DEAs, such as DEA 6 and DEA 8, but typing antisera for these antigens no longer exist, so these DEAs are therefore not considered when discussing DEAs and their corresponding antibodies.[24] The Japanese dog blood antigen system, which differs from the Western DEA system, may also be a consideration. The Japanese system includes the blood antigen NAN and NGN group, the D1, D2, and D1D2 group, and the C type group.[24,30,37,56,57] The NAN positive dogs were shown to express an anti-NGN antibody.[24,30] It is unknown if a corresponding DEA to the NAN/NGN system exists. Within the D system, antigen D1 corresponds to DEA 3, but D2 does not have a DEA counterpart. Studies on the repeated transfusion of D2 into a negative recipient have resulted in transfusion reactions.[24] The Type C system also differs from the DEA system.

Inconsistent results may also be attributed to antibodies that may react with the red blood cell non-specifically. Such antibodies may include anti-red blood cell membrane (anti-RBCm) antibodies associated with stored red blood cells, a possible anti-dog lymphocyte antigen (DLA) antibody, or a possible anti-complement antibody.[58-60] The significance of anti-RBCm was demonstrated by

Adachi et al. (1994) in the study of *Babesia gibsoni*-infected dogs.[61] The infected dogs' red blood cells were damaged by *B. gibsoni* and the damaged cells became reactive with anti-RBCm that was present in the dog before infection. All red blood cell donors utilized in this study were disease-free, and therefore not infected by *B. gibsoni*. However, if the red blood cells utilized in this study were damaged prior to screening, for example, by improper storage, perhaps an anti-RBCm present in the serum sample could react with the damaged cells and give a positive result. Other possible non-specific antibody interactions are modeled after human transfusion medicine principles. The American Association of Blood Banks Technical Manual, 15th Edition describes antibodies that are reactive with red blood cells that have been stored.[11] Such antibodies are not removed with cell washing. The red blood cells utilized in this study were stored for up to 34 days, although most were used within 15 days, as described in the Materials and Methods section. If a similar antibody to stored red blood cells exists in the dog, it may explain some spurious results. The AABB Technical Manual also describes an anti-human lymphocyte antigen (HLA) antibody in the human which may react with HLA expressed on the red blood cell. If the dog produces an anti-DLA antibody and expresses DLA on the red blood cell[62], this may explain some inconsistent results. A final consideration in non specific red blood cell reactivity is the possible anti-complement antibody. The human model demonstrates the existence of such an antibody with the Chido/Rodgers group.[11] Such an antibody is a possibility and could result in positive reactions during testing, but it would not be of clinical significance in the animal.

Further possible explanations for spurious reaction patterns may include causes related to antigens or antibodies, but unrelated to specificity. One possible explanation for inconsistent results is inappropriate antigen-antibody ratio resulting in prozone or postzone. This ratio of red blood cell suspension to serum was controlled in this study, however, the titer of antibody in each individual serum sample could not be controlled. Another explanation may be that antibodies may not react with all corresponding antigens. Also, weak antibody reactivity or low antibody titer may be a factor.[14] The variation in antigen expression over time may also be a factor in differing reactivity. If the antigen had been expressed strongly during one phase of testing, the results may have been very distinct. However, if the antigen expression was reduced over time, the results may have become less distinct or non-existent. The Positive Control did not demonstrate a change in reaction strength during the course of this study, which suggests there was not a change in the level of antigen expression for the DEA targeted by the Positive Control serum. Other DEAs may have fluctuated in level of expression, although that cannot be determined for certain.

The age and condition of the serum samples included in this study must be considered. All of the samples were submitted to MABS from various laboratories and it is impossible to determine how each sample was handled prior to submission to MABS. Storage methods may also be a consideration. Young et al. (1952) described a loss of antibody activity after storing serum for 24 hours at 4°C.[15] Other studies have also documented the decrease or loss of

antibody activity during storage at 4°C.[14,48,63] These studies demonstrate the loss of antibody activity with storage, which could lead to samples being considered negative despite the original presence of reactive antibodies. The findings in these studies may be a consideration when evaluating samples that are of unknown background, however, the previous studies are more than 45 years old. There have not been more recent studies documenting such storage effects.

Strott et al. (1969) reported a loss of antibody potency with repeated cycles of freezing and thawing of serum.[64] It is suspected that this freezing and thawing procedure may have an impact on the antibody activity in serum. The effect of such activity on antibody reactivity is of concern in this study because the samples were subjected to repeated cycles of freezing and thawing to accommodate inclusion in both the Detection and Identification Screens. Samples were subjected to at least two freezing and thawing cycles during the study, but may have been subjected to up to four freezing and thawing cycles due to a miscommunication early in the study. This procedure may explain the reaction pattern observed in the 62 samples that reacted in the Detection Screen but failed to react in the Identification Screen.

The impact of the age and quality of the red blood cells in this study must also be considered. As the red blood cells are stored, the antigens may deteriorate, thereby giving inconsistent results over time (AABB).[11] The AABB Technical Manual indicates that serologic testing reactions may be anomalous as the red blood cells age.[11] Older red blood cells and red blood cells damaged by

some other factor (e.g. improper storage) may have been less capable of surviving the multiple-step screening process. Additionally, Olson (1940) suggested that dog red blood cells are more fragile than human red blood cells.[48] The inherent fragility of the dog red blood cell may also have reduced the capability of the cell to survive the screening process. The Positive Control consistently survived the multiple-step screening process, however, cell buttons were often smaller in size by the end of the process.

Several non-immune related factors may have impacted the results in this study. Rouleaux formation may be a possible source of false positive reactions, as documented by previous studies.[15,63,65] Although rouleaux formation is a possibility, it is less likely due to the cell washing procedure prior to testing. This preparation of the cells would have removed the proteins responsible for rouleaux formation from the red blood cell samples. Proteins in the serum samples may still have been a factor in rouleaux formation, however, such formations would have disassociated in the ACG phase. A more likely source of false positive reactions is the particulate matter in the serum samples, which may give the appearance of agglutination when red blood cells become trapped in the matter. Although the samples were centrifuged prior to testing, it is still possible that particulate matter was transferred to the tubes for testing. Particulate matter was viewed, on occasion, in the tubes after the serum was dispensed. A source of possible false negative is the loss of red blood cells during the screening process. The multiple-step screening process may have led to the destruction of fragile red blood cells, some of which may have had bound antibody on the

surface. In those cases, the loss of antibody-bound cells may have caused the reaction to be missed and falsely called negative. A previous study by Olson (1940) indicated that partial hemolysis in a sample of red blood cells may decrease the ability or tendency for agglutination in the remaining cells.[48] Hemolysis was included in the observations for this study, but if the destruction of cells occurred during washing, it would have been missed. Finally, a source of false results, either positive or negative, may have been caused by technical error during the screening process.

Considerations in this study

Problems arose related to the DEA type of two of the screening dogs included in this study. The DEA 1.3,4,w7 positive dog expressed DEA 7 weakly when compared to the expression of DEA 7 on other dogs. As a result, it is unknown if this dog may have produced false negative reactions in the presence of anti-DEA 7 due to the reduced expression of the antigen. The second problem arose with the DEA negative dog. A true DEA negative dog should not have reacted with any frequency in the presence of dog serum, but this particular dog reacted 29 times during the 184 tests (15.8%). These numbers suggest that the dog does express either some type of DEA, perhaps DEA 8, or reacted due to one of the factors previously mentioned. Typing antisera for DEA 8 no longer exist, so it is currently impossible to identify the antigen on a dog's red blood cells. However, past studies indicated a 40 to 45% prevalence of DEA 8 in the random dog population.[24] The DEA Negative dog may be negative for all

antigens that are currently identifiable, but may express an antigen, such as DEA 8, that cannot be identified with available reagents. When the DEA negative dog is removed from the screening results, 30 of the samples with unknown specificities fall into patterns with defined specificities, giving a total of 92 samples with specificities (Table 14). This suggests that the reaction patterns for the 30 samples with newly assigned specificities were present originally, but could not be determined due to the DEA Negative dog reaction. The new specificities for the 92 samples are as follows: 17 samples were anti-DEA 3; 10 samples were anti-DEA 5; 25 samples were anti-DEA 7; and 39 samples were multiple antibody combinations. Only 31 samples remained with an unknown specificity.

Removing the DEA Negative dog from the red blood cell panel resulted in different p-values for each DEA (Table 15). DEA 1.1 and DEA 5 have p-values of 0.05 or less when calculated by either the Fisher Exact or the Harris and Hochman method, and therefore remain within the 95% confidence interval. DEA 3 and DEA 7 have p-values of less than 0.05 when calculated by the Harris and Hochman method but not by the Fisher Exact method. DEA 1.2, DEA 1.3, and DEA 4 do not have p-values of less than or equal to 0.05 when calculated by either the Fisher Exact or the Harris and Hochman method, therefore do not maintain the 95% level of confidence.

Ideally, the samples that produced spurious results or were excluded from this study would have been rescreened to verify the results. However, many of the red blood cell donor dogs used in this study were no longer accessible for

Table 14: Reaction patterns of screening dogs after removing the DEA Negative dog from the results

Dogs Reacting						Antibody Specificity	Number of Additional Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA 3,4,5		
+	0	0	0	+	0	unknown	1
+	0	0	0	0	+	anti-3	3
+	0	0	0	+	+	anti-5	2
0	0	+	0	+	+	unknown	1
+	+	0	0	+	+	anti-3,7 anti-5,7 anti-3,5,7 anti-1.2,5 anti-1.2,3,5 anti-1.2,3,7 anti-1.2,5,7 anti-1.2,3,5,7	6
+	+	0	+	+	+	anti-1.1,3,7 anti-1.1,5,7 anti-1.1,3,5,7	5
0	+	+	+	+	+	anti-1.1,3,7 anti-1.1,5,7 anti-1.1,3,5,7	1

Table 14 (continued)

Dogs Reacting						Antibody Specificity	Number of Additional Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	**DEA 1.2,4,7**	**DEA 1.3,4,w7**	**DEA 1.1**	**DEA 1.1,4,5,7**	**DEA 3,4,5**		
+	+	+	0	+	0	anti-7	2
+	+	+	0	+	+	anti-3,7 anti-5,7 anti-3,5,7 anti-1.2,3,7 anti-1.2,5,7 anti-1.2,3,5,7 anti-1.3,3,7 anti-1.3,5,7 anti-1.3,3,5,7 (and any of these combinations with anti-4)	5
+	+	+	+	+	+	unknown	3
+	+	+	0	0	0	unknown	1
						TOTAL	30

Table 15: p-values for each DEA after removing the DEA Negative dog

DEA	# Positive	# Negative	p-value (Fisher)	p-value (H&H)
1.1	3	3	0.050	0.016
1.2	1	5	0.167	0.080
1.3	1	5	0.167	0.080
3	2	4	0.067	0.022
4	5	1	0.167	0.080
5	3	3	0.050	0.016
7	4	2	0.067	0.022

donation of blood. Additionally, the limited volume of serum samples available for screening prohibits further study of the reactivity.

When removing the questionable factors in the data, including the DEA negative dog and the samples that demonstrated reactions only during the Detection Screen, the results demonstrate clearer specificities for more of the samples (Table 16). These results were evaluated based on a total of 202 samples, which includes the 121 remaining positive samples and the 81 negative samples. These data do not reflect any possible anti-DEA 1.2 antibody due to the removal of samples that demonstrated reactions only during the Detection Screen. Any sample demonstrating only anti-DEA 1.2 would have reacted only in the Detection Screen, and would therefore be removed in this set of data. The new data set included 121 positive samples with 92 samples demonstrating a known specificity. Thirty samples (14.9%) did not demonstrate a known specificity, but 15 of the 30 samples did fall into reaction patterns that were demonstrated by 5 or more samples in each. These patterns may be indicative

Table 16: Reaction patterns of screening dogs with positive serum samples after removing the DEA Negative dog and samples that did not react in the Identification Screen from the results

Dogs Reacting						Antibody Specificity	Number of Additional Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA 3,4,5		
+	0	0	0	+	0	unknown	9
+	0	0	0	0	+	anti-3	17
+	0	0	0	+	+	anti-5	10
0	+	0	0	+	0	unknown	1
0	+	0	0	0	+	unknown	1
0	0	+	0	+	0	unknown	1
0	0	+	0	0	+	unknown	2
0	0	+	0	+	+	unknown	1
+	+	0	0	+	0	anti-7	3*
+	+	0	0	0	+	unknown	6

Table 16 (continued)

Dogs Reacting						Antibody Specificity	Number of Additional Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	DEA 1.2,4,7	DEA 1.3,4,w7	DEA 1.1	DEA 1.1,4,5,7	DEA 3,4,5		
+	+	0	0	+	+	anti-3,7 anti-5,7 anti-3,5,7 anti-1.2,5 anti-1.2,3,5 anti-1.2,3,7 anti-1.2,5,7 anti-1.2,3,5,7	15
+	+	0	+	+	+	anti-1.1,3,7 anti-1.1,5,7 anti-1.1,3,5,7	5
+	0	+	0	+	0	unknown	2
+	0	+	0	0	+	unknown	1
0	+	+	0	+	0	unknown	1
0	+	+	+	+	+	unknown	1
+	+	+	0	+	0	anti-7	22*

Table 16 (continued)

Dogs Reacting						Antibody Specificity	Number of Additional Samples Demonstrating Pattern
DEA 1.1,3,4,5,7	**DEA 1.2,4,7**	**DEA 1.3,4,w7**	**DEA 1.1**	**DEA 1.1,4,5,7**	**DEA 3,4,5**		
+	+	+	0	0	+	unknown	1
+	+	+	0	+	+	anti-3,7 anti-5,7 anti-3,5,7 anti-1.2,3,7 anti-1.2,5,7 anti-1.2,3,5,7 anti-1.3,3,7 anti-1.3,5,7 anti-1.3,3,5,7 (and any of these combinations with anti-4)	19
+	+	+	+	+	+	unknown	3
						TOTAL	121

*There are two possible patterns considered for anti-DEA 7 due to the inclusion of a dog with weakly expressed DEA 7. The weak expression of DEA 7 may lead to a negative reaction in the presence of a low titer of anti-DEA 7.

of an antibody to an unknown DEA. Overall, 17 samples (8.4%) were positive for anti-DEA 3, 10 samples (5.0%) were positive for anti-DEA 5, 25 samples (12.4%) were positive for anti-DEA 7, and 39 samples (19.3%) demonstrated a multiple antibody combination (Table 17).

Table 17: Prevalence of anti-DEA antibodies after removing the DEA Negative dog and samples that reacted in the Detection Screen but not in the Identification Screen

Antibody	Prevalence
Anti-DEA 1.1	0%
Anti-DEA 1.2	*
Anti-DEA 1.3	0%
Anti-DEA 3	8.4%
Anti-DEA 4	unlikely
Anti-DEA 5	5.0%
Anti-DEA 7	12.4%
Multiple Antibodies	19.3%
Unknown Antibodies	14.9%

* Prevalence of anti-DEA 1.2 cannot be determined because it was not represented in the Identification Screen, and would have only reacted in the Detection Screen. This pattern of reactivity was removed from the data set in this table.

Previous Studies

When considering the results of this study as compared to previous studies, the impact of screening methods must be considered. This study relied on the tube agglutination method of screening for serum antibodies and tested the serum samples at multiple phases – immediate spin, room temperature, 4°C, 37°C, and with ACG reagent. This study also defined a positive reaction as a 1+ or greater agglutination. Previous studies have utilized different techniques, such as the slide method for detecting serum antibodies, as well as tested at fewer

phases.[63] Some previous studies diluted the serum used prior to testing to avoid potential prozone.[65]

An additional consideration when comparing the results of this study with the results of other studies is the method for determining the prevalence of a reaction pattern. This study calculated the prevalence of the reaction in reference to all samples to reflect the antibody prevalence within a population. Some previous studies have utilized the same method, such as Young et al. (1952), but others, including Hale (1995) have calculated the prevalence of antibody in the DEA negative population.[12,15] The study by Young et al. (1952) reported a 2.1% prevalence of anti-DEA 3, a 9.7% prevalence of anti-DEA 5, and a 3.4% prevalence of unspecified anti-DEA antibodies in the random dog population (Table 18).[15] Hale (1995) reported the prevalence of anti-DEA antibodies as 20% anti-DEA 3 in DEA 3 negative dogs, 10% anti-DEA 5 in DEA 5 negative dogs, and 20-50% anti-DEA 7 in DEA 7 negative dogs (Table 18).[12] If the prevalence of anti-DEA antibodies reported in Hale (1995) were described in reference to a random population, as in Young et al. (1952), it is suspected the percentages would be lower.[12,15] When comparing the results of this study to studies that reported the prevalence of anti-DEA antibodies in a population of dogs negative for the corresponding DEA, consideration of this difference must be made.

Table 18: Comparison of anti-DEA prevalence in this study versus previous studies

Antibody	All Data	Adjusted Data	Previous Study *Young (1952)*[15]	Previous Study *Hale (1995)*[12]
Anti-DEA 1.1	0%	0%	0%	0%
Anti-DEA 1.2	1.9%	*	0%	0%
Anti-DEA 1.3	0%	0%	0%	0%
Anti-DEA 3	5.3%	8.4%	2%	up to 20% (in DEA 3-neg. dogs)
Anti-DEA 4	unlikely	unlikely	0%	0%
Anti-DEA 5	3%	5.0%	9.7%	10%
Anti-DEA 7	8.7%	12.4%	NR	up to 50% (in DEA 7-neg. dogs)
Multiple Antibodies	2.3-19.2%	19.3%	NR	NR
Unknown Antibodies	39.2-45.3%	14.9%	3.4%	NR
Overall	69.4%	60.0%	15%	10-50% (in DEA negative dogs)

* Prevalence of anti-DEA 1.2 cannot be determined because it was not represented in the Identification Screen, and would have only reacted in the Detection Screen. This pattern of reactivity was removed from the data set in the "Adjusted Data".
NR = Not Reported

Ideal Transfusion Medicine Practices

The human model of transfusion medicine is the most comprehensive and well-understood model in all transfusion practices. The pre-transfusion testing practices utilized in human transfusion medicine include blood typing the recipient and donor for ABO and Rh antigens, antibody screening the recipient's serum for unexpected antibodies, crossmatching the recipient and donor, and screening the donor for infectious disease.[11]

As previously described, there is no standard or regulatory requirement for pre-transfusion testing in the dog model. Suggested guidelines exist for proper

testing, donor selection, and transfusion practices; however the actual procedure followed is at the discretion of the individual veterinarian. Despite the variability in dog transfusion practices, the safest protocol for transfusion would ideally be modeled after the practices in human transfusion medicine and include donor and recipient DEA typing, recipient antibody screening, crossmatching, and donor infectious disease screening.[21,42,63] DEA typing and antibody screening is usually not performed in most veterinary hospitals, but rather, testing is usually done by reference laboratories. It is impractical to rely on reference laboratory testing of the recipient in emergency situations. The extensive pre-transfusion testing protocol would be better utilized in the case of planned surgery in which a transfusion may be necessary.

As an alternative to waiting for blood typing results from reference laboratories, many hospitals currently use the DEA 1.1 typing card to determine whether the recipient is positive or negative for DEA 1.1. The use of the card test allows veterinarians the opportunity to use donors that are DEA 1.1, 4 positive for recipients that are positive for DEA 1.1, rather than relying only on universal donors (only DEA 4 positive). Without card testing, a hospital would likely need to have access to units of blood that are universal DEA type (only DEA 4 positive).[15]

The importance of antibody screening can be seen in many different transfusion scenarios. In the case of the transfusion of antigen-positive red blood cells into an antigen-negative recipient with a pre-existing antibody to the antigen, the antibodies may lead to the clearance of the red blood cells that have

been transfused.[11] Anti-DEA 3, anti-DEA 5, and anti-DEA 7 have previously been described as non-hemolytic antibodies, but are capable of clearing red blood cells within 3 to 5 days.[12] In a recipient that is capable of regenerating its own blood cells, this clearance may be of limited significance.[11] In a recipient with regenerative capabilities that have been compromised, the clearance of transfused red blood cells is a more significant problem.[11] If the pre-existing antibodies are hemolytic, the antibodies may lead to a severe hemolytic transfusion reaction, which would be significant to any recipient.[11]

Transfusion of incompatible red blood cells may also lead to the sensitization of an antigen-negative recipient.[11] As with the transfusion of DEA 1.1 into a DEA 1-negative recipient, the initial transfusion may cause limited symptoms, but subsequent transfusions with DEA 1.1-positive blood may result in severe hemolytic transfusion reactions.[12,24,33,34]

Antibodies may also be of concern in plasma transfusions. Administering a unit of plasma containing antibodies to a recipient positive for the corresponding antigen may lead to the loss of the recipient's own red blood cells.[11] This may be of limited significance in a recipient capable of regenerating its own red blood cells, but it may be important in a recipient that has impaired capabilities in regenerating its own red blood cells.

The use of major and minor crossmatching may detect incompatibilities between the recipient and donor prior to the transfusion event, and as a result, increase the safety of transfusion practices in the field of veterinary medicine. With the advent of technologies such as the gel tube major crossmatch kit,

crossmatching procedures are becoming easier and more practical for veterinary hospitals. To prevent future incompatibilities, crossmatching should become a standard procedure prior to transfusion in dogs.

CONCLUSION

Early studies by Young et al. on the prevalence of anti-DEA antibodies in dog serum indicated a low prevalence of naturally occurring antibodies in the random dog population.[14] The study indicated a prevalence of antibody in 15% of the random dogs tested.[14] More recent studies have indicated a much higher prevalence of anti-DEA antibodies, ranging from 10% up to 50% occurrence in dogs that are negative for the corresponding DEA.[12] This study demonstrated a high level of reactivity in dog serum samples, with 69.4% of all samples tested demonstrating reactivity. Even when considering only those reactions of a 2+ strength or greater, 61.1% of all samples remained positive, suggesting that the variance in definition of a "positive" reaction alone is not a sufficient explanation for the difference in prevalence observed in this study.

Prevalence of individual known anti-DEA antibodies within the total population of samples screened appears to be 1.9% anti-DEA 1.2, 5.3% anti-DEA 3, 3.0% anti-DEA 5, and 8.7% anti-DEA 7. When multiple antibodies are accounted for, the prevalence of anti-DEA 1.2, anti-DEA 3, anti-DEA 5, and anti-DEA 7 are likely to increase, but the exact numbers cannot be determined due to the large number of multiple antibody combinations that are possible. There was no occurrence of anti-DEA 1.1 or anti-DEA 1.3, consistent with the findings of previous studies which indicated that anti-DEA 1 group antibodies do not occur naturally.[12,13,24,33] Anti-DEA 4 is unlikely to be present in the serum samples due to the high prevalence of DEA 4 in the dog population. Many reactions were of an unknown specificity, and may represent antibodies to DEA groups not defined

on the red blood cells utilized in this study, unusual combinations of antibodies, or non-specific antibodies.

When removing the DEA negative dog from the Identification Screen panel of red cells and removing samples that demonstrated reactivity in the Detection Screen but failed to react in the Identification Screen, the prevalence of anti-DEA antibodies changes. In relation to a population of 202 serum samples, which reflects the adjusted number of positive samples (121) and the number of negative samples (81), 8.4% of samples demonstrated anti-DEA 3, 5.0% demonstrated anti-DEA 5, 12.4% demonstrated anti-DEA 7, and 19.3% demonstrated patterns consistent with multiple antibody combinations. These data do not reflect possible anti-DEA 1.2 antibodies due to the exclusion of samples that reacted in the Detection Screen but did not react in the Identification Screen. A sample positive for anti-DEA 1.2 would have demonstrated such a reaction pattern. Anti-DEA 1.2 antibodies have not been reported to occur naturally, although agglutinating antibody can be induced when an DEA 1.2-negative recipient is exposed to DEA 1.2.[24,34] Since the serum samples included in this study were from dogs with unknown medical history, antigen exposure through previous pregnancy, transfusion, or other route cannot be excluded as a possibility.

The high prevalence of anti-DEA antibodies in dog serum should be a consideration during pre-transfusion testing or selection of a donor. In humans, antibodies are often considered "clinically significant" only if they react at 37°C.[11] Warm-reacting antibodies are very important because they are most likely to

react at body temperature. Considering a sample insignificant due to a cold reaction, however, does not guarantee that the sample will not react in the body. Cold agglutinins can cause reactions in the body if the patient is exposed to cold temperatures.[11] The pattern of reactivity, as well as the temperature of reactivity, should be considered when evaluating antibody reactions.

Previous studies have suggested that naturally-occurring antibodies are not a significant concern in the dog.[51] In a recipient that is capable of regenerating its own red blood cells, anti-DEA antibodies may not be of significant concern. However, in the recipient that is incapable of fully regenerating its own red blood cells, the loss of red blood cells due to antibody interaction may be a great concern. Antibodies with hemolytic properties, including acquired antibodies and, possibly, undefined naturally-occurring antibodies, present a bigger problem for the recipient. These antibodies can lead to hemolytic transfusion reactions, which may lead to broader systemic manifestations, including renal failure or shock.[11,20-23]

This study demonstrated a high level of *in vitro* reactivity in the dog serum samples. Antibodies capable of reacting at only cold temperatures may not be significant in the dog, however, many antibodies detected in this study were capable of reacting at both cold and warm temperatures. Since the samples were collected mostly from potential donor dogs, the study's overall transfusion significance for the dog population may lie in the prevalence of antibodies in donated plasma. Antibodies administered to a recipient through plasma transfusion are also capable of causing a transfusion reaction. If the antibodies

are hemolytic, they may destroy the recipient's red blood cells and the transfusion reaction may be severe and cause physical symptoms of incompatibility. If the antibodies are not hemolytic, they may still lead to the clearance of the recipient's red blood cells, which may be significant to a recipient with limited red blood cell regenerative capabilities.

The high prevalence of antibodies may also be of concern in a recipient receiving a red blood cell transfusion. If the recipient has preexisting antibodies, either naturally-occurring or acquired, to an antigen on the transfused red cells, the recipient's antibodies may clear or destroy the transfused red blood cells, depending on the antibodies involved. The significance of the reaction may depend on the status of the recipient and the severity of the transfusion reaction.

Screening for antibodies against DEAs is important because this study, as well as previous studies, has demonstrated the prevalence of anti-DEA antibodies. Although previously reported naturally-occurring anti-DEA antibodies, such as anti-DEA 3, anti-DEA 5, and anti-DEA 7, have been described as non-hemolytic, they may still be of significance in the recipient. Undefinable anti-DEAs may be of greater concern, since it is unknown how they may react *in vivo*. Acquired antibodies also present a potential risk, because they may cause hemolytic transfusion reactions as seen with anti-DEA 1.1 or anti-DEA 4.[33,38] Ultimately, the level of significance depends on the health condition of the individual receiving the transfusion and the immune factors involved with the transfusion. However, recipients of either plasma or red blood cell transfusions are often of compromised health status. To ensure the safety of

transfusions in the dog, antibody screening, as well as major and minor crossmatching, should be routinely performed.

Areas for future studies include serum antibody screening focused specifically on the prevalence of anti-DEA antibodies among dogs of specific breeds, geographic regions, and dogs with histories of antigen exposure by previous pregnancy, transfusion, or transplant.

APPENDIX

Alsever's Solution

20.5 grams (g) dextrose, 4.2 g sodium chloride, 0.55 g citric acid · H_2O, and 8.0 g citric acid (Tri sodium salt) were added to double distilled water (ddH_2O) to a volume of approximately 1 liter. The solution was corrected to pH 7.4 using 1 molar (M) HCl or 1M NaOH and brought up to a final volume of 1 liter with ddH_2O. The solution was bottled and autoclaved. Unopened bottles were stored at room temperature for up to 6 months. Opened bottles were stored at 4°C for up to 1 month.

ACG Reagent

Anti-Dog IgG (whole molecule), developed in rabbit, delipidized whole antiserum (Lot.062K4883, Sigma-Aldrich Co.) was titered to evaluate activity of the reagent. A dilution of 1:50 in PBS was determined as the ideal working concentration. Dilutions were aliquotted in 1.5 ml volumes in microcentrifuge tubes and were stored at -70°C.

Check cells

Check cells, or sensitized red blood cells, were prepared to verify the viability of the Coombs reagent. Canine red blood cells from the donor with blood type DEA 1.2, 4, 7 were washed three times with PBS. One hundred μl of washed cells was combined with 150 μl of serum containing anti-DEA 4 antibodies and was incubated at 37°C for 1 hour. The sensitized cells were washed three times with PBS. Thirty μl of washed sensitized cells were resuspended in 970 μl PBS

to prepare a 3% check cell suspension. Fresh check cells were prepared each day of testing.

Phosphate Buffered Saline (PBS)

32.0 g NaCl, 0.8 g KCl, 4.6 g NaH_2PO_4, and 0.8 g KH_2PO_4 were added to ddH_2O to a volume of approximately 1 liter. The solution was corrected to pH 7.4 using 1 molar (M) HCl or 1M NaOH and brought up to a final volume of 4 liters with ddH_2O. The solution was bottled and stored at room temperature for up to 6 months.

Prior to using, the new lot of PBS was quality control tested by the hemolysis test in parallel with the previous lot of PBS and the lab stock of PBS. The hemolysis test consisted of adding 1 ml washed packed red blood cells to 5 ml PBS, mixing thoroughly, and incubating at 37°C for 30 minutes. The samples were centrifuged at 1200 x g for 10 minutes and were evaluated for hemolysis. Only PBS that showed no signs of hemolysis was used for testing.

Water, ddH_2O

Deionized distilled water (ddH_2O), NANOpure II system, Barnstead.

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