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Detection of Canine Immunohematologic Reactions by Gel Immunochromatography Using Immunoglobulin Binding Proteins and Anti-Canine Globulin

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DETECTION OF CANINE IMMUNOHEMATOLOGIC REACTIONS BY GEL IMMUNOCHROMATOGRAPHY USING IMMUNOGLOBULIN BINDING PROTEINS

By

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ABSTRACT

DETECTION OF CANINE IMMUNOHEMATOLOGIC REACTIONS BY GEL IMMUNOCHROMATOGRAPHY USING IMMUNOGLOBULIN-BINDING PROTEINS AND ANTI-CANINE GLOBULIN

By

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Detection of erythrocyte sensitization using a gel column can be beneficial to veterinary immunohematology. Compatibility testing is recommended for canine transfusion, but the traditional test tube method can often be difficult to interpret and prone to variation depending on the skill of the individual performing test. A gel method can ease interpretation and reduce variation. Agarose gels were purchased containing bacterial proteins A, G, and L, and an anti-canine globulin gel was created by adhering whole molecule rabbit anti-canine globulin to a Cyanogen bromide activated agarose gel. Aliquots of each gel, with erythrocytes sensitized with antibodies to dog erythrocyte antigens (DEA) 1.1, 1.X, 3,5, 4, 5, and 7, were centrifuged at 1200 gravities for 10 minutes. The results were compared to those obtained by the traditional test tube method. Gel columns with anti-canine globulin and columns without immunoglobulin binding protein proved to be effective for detecting cells sensitized to DEAs 1.1, 4, and 5, and in some cases, better than the test tube method. This method provides easy interpretation, which is helpful for canine pre-transfusion testing.

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LIST OF ABBREVIATIONS

Anti-Canine IgG.....ACG

Dog Erythrocyte Antigen.....DEA

Immunoglobulin A.....IgA

Immunoglobulin G.....IgG

Immunoglobulin M.....IgM

Institutional Animal Care and Use Committee..... IACUC

Midwest Animal Blood Services.....MABS

Registered Trademark.....®

United States Department of Agriculture..... ..USDA

Introduction

Traditionally, test tube methods are used to detect agglutination for human compatibility testing and typing; however, gel methods have gained popularity due to their sensitivity, ease of performance and interpretation, and stability of results. In commercially available human immunochromatographic gel cards, immunoglobulin binding proteins that can bind to sensitized human erythrocytes are adhered to gel particles (like Sepharose®). It is known that these immunoglobulin binding proteins can bind to canine immunoglobulins. An immunochromatographic method for detecting canine immunohematologic reactions could prove to be beneficial.

Gel cards are also available for human erythrocyte antigen typing. These cards also contain gel particles. However, they contain anti-sera to the specific antigen instead of a bacterially derived immunoglobulin binding protein.

Compatibility testing in humans is performed for all patients receiving a transfusion with the exception of extreme emergencies when time does not permit.[1] Its purpose is to detect any testing errors due to ABO (significant, naturally occurring antibodies) incompatibility and to a lesser extent incompatibilities due to unexpected antibodies (antibodies due to pregnancy or previous transfusion).[1] Compatibility testing in dogs, although recommended and beneficial to the recipient [2], may not be as frequently performed primarily because incompatibility reactions to single transfusions are rare, and multiple transfusions are not as frequently performed in veterinary medicine so there is less concern over acquired antibodies. There are also no required standards for

veterinary pre-transfusion testing. The consequences of transfusing an incompatible unit greatly range in severity. A transfusion with an incompatible unit may be as harmless as being asymptomatic or, may lead to something more severe, like death in humans.[3] Although less information is available on the effects of transfusing incompatible blood in dogs, the effects have a potential for being severe.[2]

This study compared a modified gel test method, in a microcentrifuge tube, to a traditional test tube method for the detection of canine immunohematologic reactions. The gel method is not currently used in detecting canine erythrocyte sensitization, but it may lead to a less complex and more consistent method for typing and determining compatibility of canine blood. Gels with different bound proteins were compared to gel without protein and to the test tube method to determine the effectiveness of a gel test method for dogs.

Literature Review

Transfusion History

Some of the initial accounts of transfusions involve dogs as donors and/or recipients. These early transfusions were not for therapeutic purposes in dogs, but experimental for the benefit of humans. The objective was to determine if inter-species transfusions or intra-species transfusions were safe and perhaps had healthful results. Karl Landsteiner's discovery of the human ABO blood group in 1900 led to early methods of pre-transfusion testing.[4] As both veterinary and human transfusion medicine developed better equipment and techniques, therapeutic transfusion for dogs became possible.[4] In the 1960's [5] and 1970's, canine blood groups were first described; however, typing reagents were limited because few laboratories were interested in producing typing sera. The leading producer and distributor of canine typing sera was Dr. Robert Bull's laboratory at Michigan State University, East Lansing, MI.[4]

Basic immunohematology

Immunohematology focuses on the detection of immune responses to antigens on blood cells.. These antigens comprise proteins, carbohydrates, and/or lipids. Methods for detecting *in vivo* and *in vitro* immune reactions involve detecting sensitization of blood cells, i.e., antibody binding to blood cells. The focus of this discussion is on erythrocyte immunohematology.

For antibodies to react, certain conditions must be met. Antibodies, frequently immunoglobulin M (IgM), formed against carbohydrate antigens tend

to form stronger bonds at lower temperatures; conversely, antibodies, typically immunoglobulin G (IgG), to protein antigens tend to form stronger bonds at higher temperatures.[6] Conditions, other than temperature, such as pH, reaction time, and ionic strength of the solution are equally important.[6] The optimal pH is species and immunoglobulin class dependent. Antibodies may interact with erythrocyte antigens and form a complex by binding antigens on more than one cell. The visible lattice that is formed is referred to as agglutination.[6] For antibodies to be able to encounter the antigen, the correct ratio of antibody to antigen is necessary; for this reason, packed erythrocytes are often diluted to 2-4 percent (%) solutions. Optimally, antigens will not be exposed to too many antibodies making cross-linking less likely, or too few antibodies, leaving many erythrocytes unsensitized or undersensitized and thus show decreased visible agglutination.

Testing methods are usually designed to mimic body conditions, to prevent reactions that are not clinically significant (they do not occur under body conditions). Thus, testing is frequently performed at normal human body temperature 37 degrees Celsius (°C). There are also instances when incubating at room temperature or 4 °C is recommended. These conditions often favor the reaction of particular antibodies (usually IgM) *in vitro*.[6]

Reactions are evaluated by visually checking for the presence or absence of agglutination. Frequently, antibody or complement will be present on an erythrocyte, but due to the antigen sites' locations and density agglutination does not occur.[6] In such cases, sensitization is detected by adding a secondary

antibody. In human immunohematology, this antibody is anti-human globulin (AHG), anti-C3d (a component of the complement system), or a combination. This antibody, if it is AHG, will bind the primary antibody or, if it is anti-C3d, complement (already bound to the cell), forming a bridge to other cells, aiding visible agglutination.[6] In canine immunohematology, the antibody is anti-canine IgG (ACG). This will bind the canine IgG if present.

Of the five classes of antibodies, IgM and IgG are the most significant because they are present in the serum in significant quantities [7], and are frequently formed against erythrocyte antigens.

IgM exists as a pentamer containing ten antigen binding sites; however, only about five are readily available. Because it is multivalent, IgM tends to form more visible agglutinates (erythrocytes cross-linked with antibodies to form clusters) when exposed to erythrocyte antigens. It is the initial antibody produced in response to foreign antigen. IgM can also help activate complement, which may lead to lysis instead of agglutination.[7]

IgG is the most prevalent antibody in the sera. It is present only as a monomer.[7] Most subclasses of human IgG can activate complement as well as cross the placenta.[7] IgG on the surface of foreign particles enhances interactions with phagocytes.[7]

Current practices in veterinary and human immunohematology

Tube testing methods have traditionally been used in human immunohematology. They involve *in vitro* sensitization of erythrocytes using a 2-

4% suspension of erythrocytes to which serum is added. The reaction is allowed to take place within a test tube at optimal temperature. The temperature selected is dependent on the antibody class for the blood group being observed. The test tube is centrifuged, gently shaken until the cell button detaches from the bottom of the test tube, and the reaction is graded based on the degree of agglutination, which is assessed visually using an optical aide. The veterinary field also uses this method for pre-transfusion testing. A major flaw of this test is the variation due to the quality of the performer's technique. The more modern gel test minimizes variation and, according to manufacturers, has the advantage of increasing productivity, standardization, and compliance with regulations.[8] Other sources agree, saying it offers the benefits of decreased labor, consistent results, and a stable reaction which may be reviewed at a later time.[9]

In the human gel test, a gel is created with Protein A and/or G, bacterially derived immunoglobulin binding proteins, adhered to the gel particles.[10] The gel is also available with AHG adhered to the gel particles. The gel, whether bound with proteins A and G or AHG, is placed in wells (similar to microcentrifuge tubes) in a card. A 0.8% red blood cell solution from the donor is incubated at optimal temperature with serum or plasma of the recipient in the wells of the card. Next, the card is centrifuged and interpreted using visual comparison to a chart. The gel-adhered protein binds the Fc portion of immunoglobulins, trapping agglutinates in the gel, making them easy to interpret. A 4+ reaction appears as a population of cells at the top of the gel column. Negative reactions appear as a delineated population of cells at the bottom of the reaction well. Any agglutinates

captured within the gel denote a positive reaction and are graded according to how far down the gel they are captured.[10]

Antigen typing can also be performed by modifying this method. A mixture of gel and anti-sera is within the well of the card. The unreacted erythrocytes are placed on top of the gel and centrifuged. During centrifugation, the cells will react with the anti-sera, agglutinate, and be captured within the gel.[10] Unlike the tube method, reacted erythrocytes do not need to be washed for this test since there is a matrix above the gel responsible for keeping unbound (free) antibody from traveling through the matrix and binding to the immunoglobulin binding proteins, thus inhibiting the sensitized cells from being bound. This reduces the time it takes to perform compatibility testing, antibody screening, or antigen typing. This test is more standardized and more sensitive than its tube counterpart.[4] However, it can be more expensive than the tube method due to the initial investment in special equipment to incubate and centrifuge the cards, as well as the regular purchase of gel cards and their corresponding diluents. The true cost of this method can only be determined by accounting for technologist time, number of samples being tested, and the extent to which the cards are being used.[11]

During this study, a simplified gel test became available to veterinary clinics. This newer test, available from Midwest Animal Blood Services (Stockbridge, MI), utilizes a gel matrix without immunoglobulin binding proteins in a microcentrifuge tube. The sieving properties of the gel particles are responsible for capturing agglutinates in a positive reaction.

Another advantage human immunohematology has over veterinary immunohematology is the availability of relevant monoclonal antibodies. Monoclonal antibodies, often from murine sources, are readily available against many human erythrocyte antigens. These antibodies provide consistent specificity for, and strong reaction to, their corresponding antigen.[7, 12] Due to the very high specificity of monoclonal antibodies, multiple different ones are often mixed together.[7] This mixing allows for more than one class or subclass of antibody to be detected. Monoclonal antibodies to dog erythrocyte antigen (DEA) 1.1 [13] and 4 [12] have already been created. A card agglutination test has been developed using monoclonal anti-DEA 1.1.[13] Other monoclonal reagents, A, B, D, and E, have also been developed in Japan. Reagent A is thought to be equivalent to anti-DEA 3.[14]

DEA System

Nomenclature for canine blood groups has changed over the years. The current system used in the United States was proposed in 1976.[15] Canine blood groups are based on the DEA nomenclature system. In this system, there are seven clinically significant DEAs: DEA 1.1, 1.2, 1.3, 3, 4, 5, and 7. The chemical structures of these antigens are not known. Exposure to these erythrocyte antigens may lead to a transfusion reaction in antigen-negative dogs.[16] Antibodies responsible for eliciting the transfusion reaction may be produced innately without exposure to an antigen; these are termed expected antibodies. Conversely, the antibodies may be produced following exposure to a foreign

antigen, usually from previous transfusions or pregnancy; these are termed unexpected antibodies. Some antigen-negative dogs may produce naturally occurring antibodies (anti-) to DEA 3, 5, and 7, but most canine blood groups are not known to have naturally occurring antibodies. Dogs are known to become sensitized by pregnancy or transfusion to DEA 1.1, 1.2, 1.3, 3, 4, 5, and 7.[16, 17, 18] DEA 1.1 and 1.2 are known to cause acute hemolytic transfusion reactions.[16]

The blood group system DEA 1 contains DEAs 1.1, 1.2, 1.3, and null alleles.[19] Inheritance is likely autosomal dominant.[19] Exposure of a previously exposed DEA 1.1 negative dog to DEA 1.1 positive erythrocytes may lead to rapid adverse hemolytic transfusion events.[16] This is also true of DEA 1.2; however, it typically takes longer for erythrocyte removal.[16] Both DEA 1.1 and 1.2 are known to be proteins. Less is known about DEA 1.3 because it is the most recently recognized antigen in this group. Immunization of a null dog with DEA 1.3 leads to antibodies that react against DEAs 1.1, 1.2, and 1.3.[20]

Dog erythrocyte antigens 3 and 5 are low incidence antigens.[16] They have been known to cause delayed hemolytic transfusion reactions.[16] In both the DEA 3 and 5 systems, dogs can have the antigen or the null phenotype.[19] Naturally occurring antibodies to DEA 3 occur in up to 20% of the DEA 3 antigen negative dogs.[17] Within 5 days after a transfusion of DEA 3 positive erythrocytes, dogs with antibody to DEA 3 experience erythrocyte destruction.[17] Ten percent of randomly sampled dogs in the United States have naturally occurring antibody to DEA 5.[17] Dogs producing this antibody, which have been

transfused with DEA 5 positive cells, experience erythrocyte destruction within 3 days.[17]

Dog erythrocyte antigen 4 is thought to be a protein of about 32-40 kDa.[21] There are two phenotypes, DEA 4 and null.[19] The significance of antibodies against DEA 4, a high incidence antigen, in transfusion is debatable.[16] Early erythrocyte survival studies showed no cell loss or hemolysis.[17] However, naturally occurring antibodies have been identified.[22]

The DEA 7 system, also known as the Tr systems, has three phenotypes, Tr, O, and null.[19] Dog erythrocyte antigen 7 negative dogs may have a naturally occurring antibody that may cause a delayed non-hemolytic transfusion reaction. The significance of this reaction is dependent on the recipient's ability to regenerate its own erythrocytes.[16] It has been suggested that this antigen is adsorbed onto erythrocytes from the serum.[23]

Gel

The gel method for detecting immunohematologic reactions was originally developed by LaPierre.[10] The method LaPierre developed starts with a card similar in size to a credit card. Within this card, there are wells (Figure 1). The top of these wells is wide and shallow; it also contains fluid to keep the gel column from drying. The area on top is provided for reagents to incubate.[10] The tube narrows and lengthens at the bottom (this area is the column). The column is where the gel is, and where the reaction is observed. Specialized incubators and centrifuges are provided for use with these commercial gel cards.[10]

In commercial gel card methods, a 0.8% erythrocyte suspension and serum (if applicable) are added directly to a well. The entire card is placed in the incubator. After incubation, the card is placed in the centrifuge and centrifuged at 70 gravities (g) for 10 min. The reaction is then graded based on the location at which the cells remain in the column. A 4+ reaction is when the cells remain at the top of the gel. The reaction strength is considered weaker the further down the column the cells settle.[10] A negative reaction is when there is a delineated population of cells at the bottom of the column (Figure 1).[24]

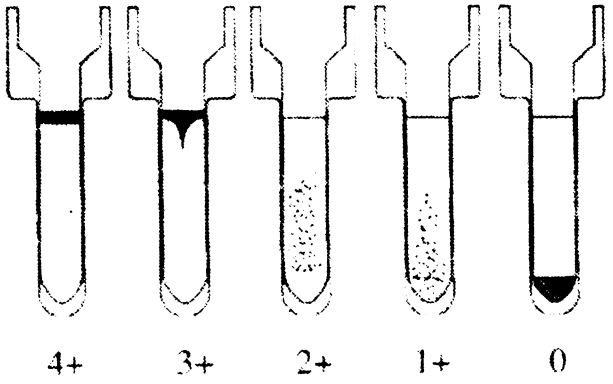

Figure 1: Interpretation guide for grading gels

The gel within this method has two functions. The first way the gel captures immunohematologic reactions is by the size of the erythrocyte agglutinates. The beads of gel act as a sieve. Larger agglutinates cannot migrate as far down the column as smaller agglutinates. The second way the gel captures immunohematologic reactions is by capturing immunoglobulins on the surface of erythrocytes using immunoglobulin binding proteins on the particles of gel. Weak gravitational forces and a long centrifuge time aid in the ability for the

immunoglobulin binding proteins and the sensitized erythrocytes to react. The cells attached to the gel are thus prevented from migrating to the bottom of the column during centrifugation.[10]

The gel column has been found to be more sensitive than the tube method at detecting IgG in direct antiglobulin testing.[25] Comparative testing between test tube and gels for indirect globulin testing also shows a high degree of sensitivity.[26]

Immunoglobulin binding proteins and anti-canine globulin

The likely purpose of bacterial cell wall proteins A, G, and L is as virulence factors. They adhere the bacteria to the host immunoglobulin's Fc region, coating the bacteria with these immunoglobulins. This enables the bacteria to avoid phagocytes, which also utilize the Fc region.

Protein A (42 kilo-Daltons; kDa) [27], is a cell wall protein isolated from *Staphylococcus aureus*. It has a wide range of possible uses due to the ability to bind to IgG of most mammals.[27] Protein A's Fc receptors are specific for certain subclasses of immunoglobulins. This leads to some limitations, but makes it a good selective reagent.[28] In dogs, protein A has been shown to bind some IgG, IgM, and immunoglobulin A (IgA).[28]

Protein G (30 kDa) is a bacterial cell wall protein originally isolated from Lancefield group G Streptococci. It has a strong affinity for IgG. This affinity is mostly toward the Fc region of the IgG molecule. Protein G is able to bind to a lesser degree to other classes of immunoglobulin due to recognition of Fab

domains.[28] Protein G has been reported to bind approximately 100% of canine IgG, 95% of IgA, and about 44% of IgM.[29]

Protein L is isolated from *Peptostreptococcus magnus* and is about 75 kDa.[30] Protein L, like the other immunoglobulin-binding proteins, has multiple copies of immunoglobulin-binding domains; however, protein L does not bind the Fc region. Protein L interacts with the light chain (primarily kappa) [31]; therefore, it is not restricted by the class of immunoglobulin. It is known to bind the immunoglobulins of many species. Studies of human polyclonal IgG showed 55% was bound to protein L. Twenty-two percent of polyclonal cat IgG was bound to protein L.[32] Research also included other animals; mouse monoclonal antibodies in particular were studied in more detail. Protein L bound strongly (72% and 79% respectively) to monoclonal murine IgG3 kappa chains and IgG2b kappa chains.[32]

Proteins A and G bind the Fc region of IgG.[28] About 82% [33] of canine IgG and approximately 65% [33] of IgM can be bound by Protein A. Protein G has been reported to bind 100% of canine IgG and 44% of IgM.[29] The percentage of polyclonal dog IgG bound to protein L was 20%.[32] The affinity for these proteins to canine immunoglobulins supports the idea that a similar gel test is feasible for canine compatibility testing.

Whole molecule ACG from a rabbit source immunospecific for canine IgG is available. This polyspecific reagent is commonly used as a Coombs' phase reagent for DEA 1. The human counterpart to ACG, anti-human globulin, is used

not only as a Coombs' phase reagent, but also in human gel cards as an immunoglobulin binding protein.

Materials and Methods

Anti-sera and cells

The polyclonal anti-sera were from Michigan State University. The anti-sera used were anti-DEA 1.1 (lot # 898227), anti-DEA 3,5 (lot # 1093AH), anti-DEA 4 (lot Sara 02/27/02), anti-DEA 5 (lot # 51471800), and anti-DEA 7 (lot Dew 09/30/01). The erythrocytes, obtained from the canine colony at Midwest Animal Blood Services Inc. (MABS; Stockbridge, MI), were as follows: Dog A (DEA 1.1^+ and 5^+), Dog B (DEA 1.2^+, 4^+, and 7^+), Dog C (DEA 1.1^+,3^+, 4^+ ,5^+ , and 7^+), Dog D (DEA 1.3^+ and 4^+), Dog E (DEA 1.1^+), Dog F (DEA 4^+ and 5^+). See Table 1 for the complete antigen profiles. All antigen profiles were previously determined by tube agglutination. All the dogs are covered under the Animal Use Form MABS 1-04 "Production of Antibody to Dog Erythrocyte Antigens". The care and use protocols were reviewed by the Institutional Animal Care and Use Committee (IACUC) of MABS, United States Department of Agriculture (USDA) 13343.

Table 1. Antigenic profiles
"+" indicates antigen is present, and "0" indicates antigen is absent.

	DEA 1.1	DEA 1.2	DEA 1.3	DEA 3	DEA 4	DEA 5	DEA 7
Dog A	+	0	0	0	0	+	0
Dog B	0	+	0	0	+	0	+
Dog C	+	0	0	+	+	+	+
Dog D	0	0	+	0	+	0	0
Dog E	+	0	0	0	0	0	0
Dog F	0	0	0	0	+	+	0

Making a 3% erythrocyte suspension

A 3% suspension was made because it provided enough cells so that the cell layer could be visualized through the gel matrix. Approximately 500 microliters (µl) packed erythrocytes were transferred into a 12 X 75 millimeter (mm) borosilicate glass test tube. This tube was filled with phosphate buffered saline (PBS; 0.14 molar (M) NaCl, 0.003 M KCl, 0.01 M Na H_2PO_4, 0.001 M KH_2PO_4) and centrifuged in a centrifuge (Immufuge II; Baxter, Deerfield, IL) for 2 minutes (min) on high (1140 g). The PBS was vacuum aspirated leaving a cell button. The tube was again filled with PBS and centrifuged for 2 min on high. After vacuum aspiration, 75 µl of the erythrocyte button was transferred to a glass tube containing 2.425 milliliters (ml) PBS. The cells were resuspended using gentle end-over-end mixing until they were uniformly distributed.

Gel preparation

Streptococcal protein G adhered to lyophilized 4 % beaded agarose (45 to 165 micrometers (µm); P7700, Sigma, Saint Louis, MO) was stored in the freezer prior to being swollen in 30 ml of water for 30 min. The resin was rinsed with PBS and stored in storage buffer (PBS with 0.02% sodium azide) at 4 ºC.

Lyophilized protein A adhered to 4% beaded agarose gel (45 to 165 µm; P9269, Sigma) was swollen in 30 ml Buffer A (0.02 M NaH2PO4, 0.15 M NaCl, 0.015 M sodium azide) for 30 min. The resin was rinsed five times with 10 ml Buffer A and stored in storage buffer.

Protein L adhered to 4% beaded agarose gel (45 to 165 µm; P3351, Sigma), was purchased in suspension and rinsed and stored in storage buffer at 4 °C.

Lyophilized, whole molecule, anti-canine IgG (D8650, Sigma) was coupled to a cyanogen bromide activated 4% agarose (40-165 µm; C9210, Sigma) as follows: First, the ACG was dissolved in coupling buffer (0.1 M NaHCO3, 0.5 M NaCl, pH adjusted to 8.4). Each wash was removed by vacuum using a Büchner funnel with Whatman number 54 filter paper (VWR, West Chester, PA). The cyanogen bromide activated agarose was swelled and washed ten times over a period of 30 min using 20 ml of 1 millimolar (mM) HCl kept at 4 °C. The agarose resin was washed ten times using a Büchner funnel and 7 ml of deionized water per wash. The resin was rinsed once with 5 ml coupling buffer. The dissolved ACG and agarose resin were mixed in a 50 ml conical vial for 2 hours (h) using a tilting mixer at room temperature. The agarose resin, bound with ACG, was filtered using a Büchner funnel, and washed with 5 ml coupling buffer. It was blocked with 0.2 M glycine for 2 h on a tilting mixer at room temperature. After blocking, the ACG agarose was subjected to five wash cycles, each consisting of 5 ml coupling buffer followed by 5 ml acetate buffer (0.015 M sodium acetate trihydrate, 4.82 ml/L acetic acid, brought up to 1.0 liter (L) with 0.5 M NaCl). The ACG agarose was stored at 4 °C in storage buffer.

Preparation of the plain gel was identical to the ACG gel except no ACG was added to the gel. The plain gel was also stored at 4 °C in storage buffer.

Prepared gels were transferred to 0.2 ml PCR tubes (#501-PCR; Dot Scientific, Burton, MI), 200 µl per tube, and centrifuged at 1200 g for 10 min to compact the gel prior to use.

Grading gel reactions

The interpretation scheme for grading human gel cards was used as a guide to grading the gel reactions. The shape of the wells in the card and the shape of the PCR tube differ; however, a strong positive reaction (4+) resulted in all the cells remaining at the top of the gel, 3+ reactions showed cells near the top of the gel, 2+ reactions resulted in cells throughout the gel, 1+ reactions had cells within the lower half of the gel, and a negative reaction (0) resulted in a pellet at the bottom of the gel. The reaction grading is demonstrated in Figure 2.

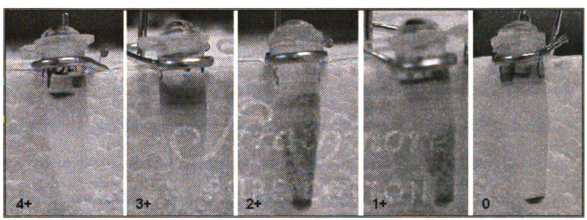

Figure 2. Grading system for gel
The full spectrum of reaction strengths is shown from strongest, 4+ agglutination on the far left, to 0 (no agglutination) on the far right.

Determining optimal microcentrifuge conditions

For every run, two 3% erythrocyte suspensions were made. The first suspension was made using cells known to express the antigen DEA 4. The

second suspension consisted of cells without DEA 4. Six reaction test tubes were set up. The first two tubes (S+) contained 50 µl anti-DEA 4 with 50 µl DEA 4 positive cells, the second two (W+) had 50 µl diluted (1/80) anti-DEA 4 with 50 µl DEA 4 positive cells, and the third two (N) had 50 µl anti-DEA 4 with DEA 4 negative cells. All six tubes were incubated at 4 °C for 30 min. One tube of S+, W+, and N were centrifuged in a centrifuge (Immufuge II; Baxter, Deerfield, IL) on high for 15 seconds (s). They were interpreted for agglutination visually using gentle shaking and observation in a mirror. The cells in the remaining three tubes were washed three times using PBS. After the last wash, the washed cells from reaction tubes were transferred to a corresponding protein A gel tube. The gel tubes were centrifuged in a microcentrifuge (Micro-centrifuge 59A; Fisher, Pittsburgh, PA).

The time and force with which the gel tubes were centrifuged differed for each run. The combinations of conditions included 900 g for 15 min, 1050 g for 10 min, 1050 g for 15 min, and 1200 g for 10 min. Once optimal conditions were determined, two more runs were performed for confirmation, and cells reacted with more dilute sera (1/160 and 1/240) were also centrifuged through gel columns to show the full spectrum of reaction strength.

Screening the gels using anti-DEA 1.1 sensitized erythrocytes

Fifty microliters of a 3% suspension of dog A erythrocytes were added to each of six test tubes labeled "pos 1.1". The same amount of a 3% suspension of dog B cells was added to five other test tubes labeled "neg 1.1". Into all of the

tubes, 50 µl of anti-DEA 1.1 was added. The tubes were incubated at 37 ºC for 15 min. After incubation one "pos 1.1" tube and one "neg 1.1" tube were centrifuged for 15 s at 1140 g. They were gently shaken and visually interpreted using a viewing aide (Agglutination Viewer model 5384; Clay Adams, Parsippany, NJ). All the tubes were filled with PBS and centrifuged at 1140 g for 1 min. The supernatant was discarded after the wash. This wash step was completed two more times. When discarding the supernatant for the last wash, excess fluid was removed using absorbent paper (Kimwipe, Kimberly-Clark, Roswell, GA) for the two tubes that were previously visually interpreted. To these two tubes, 50 µl Coombs' reagent (D7407; Sigma) was added, the tubes were centrifuged at 1140 g for 15 s, and interpreted visually. All other tubes were transferred to gels using glass Pasteur pipets. Each gel type (A, G, L, and ACG) had two tubes, a positive and a negative. These gels were centrifuged in a microcentrifuge for 10 min at 1200 g. The gels were then interpreted visually without an aide.

The entire process was completed two more times with five tubes each time. The plain tubes, one positive and one negative, were tested in the same manner as the other gels, but were only tested once.

Screening the gels using anti-DEA 1.X sensitized erythrocytes

Fifty microliters of a 3% suspension of dog B erythrocytes were added to each of six test tubes labeled "pos 1.X". The same amount of a 3% suspension of dog F cells was added to five other test tubes labeled "neg 1.X". Into all of the

tubes, 50 µl of anti-DEA 1.X was added. The tubes were incubated at 37 °C for 15 min. After incubation one "pos 1.X" tube and one "neg 1.X" tube were centrifuged for 15 s at 1140 g in a centrifuge. They were gently shaken and visually interpreted using a viewing aide. All the tubes were filled with PBS and centrifuged at 1140 g for 1 min. The supernatant was discarded after the wash. This wash step was completed two more times. When discarding the supernatant for the last wash, excess fluid was removed using absorbent paper (Kimwipe, Kimberly-Clark, Roswell, GA) for the two tubes that were previously visually interpreted. To these two tubes 50 µl Coombs reagent (D7407; Sigma) was added, the tubes were centrifuged at 1140 g for 15 s, and interpreted visually. All other tubes were transferred to gels. Each gel type (A, G, L, and ACG) had two tubes, a positive and a negative. These gels were centrifuged in a microcentrifuge for 10 min at 1200 g. The gels were then interpreted visually without an aide.

The entire process was completed one more time with five tubes due to the lack of available sera. The plain tubes, one positive and one negative, were tested in the same manner as the other gels, but were only tested once.

Unsensitized, washed dog B erythrocytes were centrifuged in an ACG gel tube to test for reactivity of the erythrocytes with the ACG gel.

Screening the gels using anti-DEA 3,5 sensitized erythrocytes

Fifty microliters of a 3% suspension of dog C erythrocytes were added to each of six test tubes labeled "pos 3,5". The same amount of a 3% suspension

of dog D cells was added to five other test tubes labeled "neg 3,5". Into all of the tubes, 50 µl of anti-DEA 3,5 was added. The tubes were incubated at 4 ºC for 30 min. After incubation one "pos 3,5" tube and one "neg 3,5" tube were centrifuged for 15 s at 1140 g in a centrifuge. They were gently shaken and visually interpreted using a viewing aide. The remaining tubes were filled with PBS and centrifuged at 1140 g for 1 min. The supernatant was discarded after the wash. This wash step was completed two more times. The tubes were transferred to gels. Each gel type (A, G, L, and ACG) had two tubes, a positive and a negative. These gels were centrifuged in a microcentrifuge for 10 min at 1200 g. The gels were then interpreted visually without an aide.

The entire process was completed two more times with five tubes The plain tubes, one positive and one negative, were tested in the same manner as the other gels, but were only tested once.

One additional run was performed entirely (including centrifugation steps) at 4 ºC to test for strict cold reactivity.

Screening the gels using anti-DEA 4 sensitized erythrocytes

Fifty microliters of a 3% suspension of dog C erythrocytes were added to each of six test tubes labeled "pos 4". The same amount of a 3% suspension of dog E cells was added to five other test tubes labeled "neg 4". Into all of the tubes, 50 µl of anti-DEA 4 was added. The tubes were incubated at 4 ºC for 30 min. After incubation one "pos 4" tube and one "neg 4" tube were centrifuged for 15 s at 1140 g in a centrifuge. They were gently shaken and visually interpreted

using a viewing aide. The remaining tubes were filled with PBS and centrifuged at 1140 g for 1 min. The supernatant was discarded after the wash. This wash step was completed two more times. The tubes were transferred to gels. Each gel type (A, G, L, and ACG) had two tubes, a positive and a negative. These gels were centrifuged in a microcentrifuge for 10 min at 1200 g. The gels were then interpreted visually without an aide.

The entire process was completed two more times with five tubes. The plain tubes, one positive and one negative, were tested in the same manner as the other gels, but were only tested once.

Screening the gels using anti-DEA 5 sensitized erythrocytes

Fifty microliters of a 3% suspension of dog C erythrocytes were added to each of six test tubes labeled "pos 5". The same amount of a 3% suspension of dog E cells was added to five other test tubes labeled "neg 5". Into all of the tubes, 50 µl of anti-DEA 5 was added. The tubes were incubated at 4 °C for 30 min. After incubation one "pos 5" tube and one "neg 5" tube were centrifuged for 15 s at 1140 g in a centrifuge. They were gently shaken and visually interpreted using a viewing aide. The remaining tubes were filled with PBS and centrifuged at 1140 g for 1 min. The supernatant was discarded after the wash. This wash step was completed two more times. The tubes were transferred to gels. Each gel type (A, G, L, and ACG) had two tubes, a positive and a negative. These gels were centrifuged in a microcentrifuge for 10 min 1200 g. The gels were then interpreted visually without an aide.

The entire process was completed two more times with five tubes. The plain tubes, one positive and one negative, were tested in the same manner as the other gels, but were only tested once.

Screening the gels using anti-DEA 7 sensitized erythrocytes

Fifty microliters of a 3% suspension of dog C erythrocytes were added to each of six test tubes labeled "pos 7". The same amount of a 3% suspension of dog D cells was added to five other test tubes labeled "neg 7". Into all of the tubes, 50 µl of anti-DEA 7 was added. The tubes were incubated at 4 °C for 30 min. After incubation one "pos 7" tube and one "neg 7" tube were centrifuged for 15 s at 1140 g in a centrifuge. They were gently shaken and visually interpreted using a viewing aide. The remaining tubes were filled with PBS and centrifuged at 1140 g for 1 min. The supernatant was discarded after the wash. This wash step was completed two more times. The tubes were transferred to gels. Each gel type (A, G, L, and ACG) had two tubes, a positive and a negative. These gels were centrifuged in a microcentrifuge for 10 min at 1200 g. The gels were then interpreted visually without an aide.

The entire process was completed two more times with five tubes. The plain tubes, one positive and one negative, were tested in the same manner as the other gels, but were only tested once.

One additional run was performed entirely (including centrifugation steps) at 4 °C to test for strict cold reactivity.

To ensure that the centrifuge and gels were working properly, a protein A gel previously centrifuged for anti-DEA 7 was reused by centrifuging with anti-DEA 4 sensitized cells.

To test for IgM activity, 450 µl of anti-DEA 7 serum with 50 µl 50 mM dithiothreitol (DTT; D0632, Sigma), and 450 µl of anti-DEA 7 with 50 µl PBS were incubated at 37 °C for 30 min. Fifty microliters of each was pipetted into separate test tubes with 50 µl 3% erythrocyte suspension of dog C. A negative control tube contained 50 µl anti-DEA 7 and 50 µl 3% dog D cells. All tubes were incubated a 4 °C for 30 minutes, centrifuged for 15 s and graded with an aide.

Results

Optimal microcentrifuge conditions

Results of the microcentrifuge study as well as the dilutions tested to demonstrate negative through 4+ reactions are shown in Table 2.

Table 2. Results of centrifuge optimization
Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). S+ = DEA 4 cells with undiluted anti-DEA 4, W+ = DEA 4 cells with 1/80 dilution of anti-DEA 4, and N = DEA 4 negative cells with anti-DEA 4.

	Test tube	900 g 15 min	1050 g 10 min	1050 g 15 min	1200 g 10 min	1200 g 10 min (run #2)	1200 g 10 min (run #3)
S+	4+	4+	4+	4+	4+	4+	4+
W+	2+	3+	3+	3+	3+	3+	3+
N	0	1+	1+	0	0	0	0
1/160 dilution	1+						2+
1/240 dilution	w+						1+

Screening the gels using anti-DEA 1.1 sensitized erythrocytes

Examples of gels A, G, L, and ACG are shown in Figure 3, Figure 4, Figure 5, and Figure 6 respectively. The results of screening gels A, G, L, ACG, control, and test tube using anti-DEA 1.1 sensitized erythrocytes are shown in Table 3.

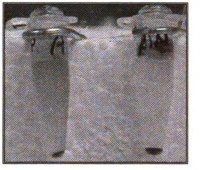

Figure 3. Protein A gels after the cells were reacted with anti-DEA 1.1 DEA1.1 positive cells are in the tube on the left, and DEA 1.1 negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.1 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

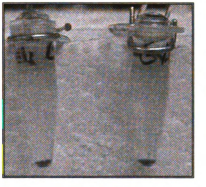

Figure 4. Protein G gels after the cells were reacted with anti-DEA 1.1 DEA1.1 positive cells are in the tube on the left, and DEA 1.1 negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.1 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

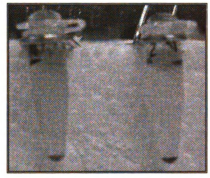

Figure 5. Protein L gels after the cells were reacted with anti-DEA 1.1 DEA1.1 positive cells are in the tube on the left, and DEA 1.1 negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.1 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Figure 6. ACG gels after the cells were reacted with anti-DEA 1.1 DEA1.1 positive cells are in the tube on the left, and DEA 1.1 negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.1 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Table 3. Results for screening effectiveness for anti-DEA 1.1 sensitized cells Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). The term "positive" refers to the tube with the cells containing DEA 1.1 antigen. "Negative" refers to the tube with cells that do not contain the antigen.

	Run #1	Run #2	Run #3
Protein			
A			
positive	1+	1+	1+
negative	0	0	0
G			
positive	0	0	0
negative	0	0	0
L			
positive	w+	w+	w+
negative	0	0	0*
ACG			
positive	4+	4+	4+
negative	0	0	0
Test tube			
positive	2+	2+	1+
negative	0	0	0
positive w/Coombs	3+	3+	3+
negative w/Coombs	0	0	0
Plain Gel			
positive	3+		
negative	0		

Screening the gels using anti-DEA 1.X sensitized erythrocytes

Examples of gels A, G, L, and ACG are shown in Figure 7, Figure 8, Figure 9, and Figure 10 respectively. The results of screening gels A, G, L, ACG, plain, and test tube using anti-DEA 1.X sensitized erythrocytes are shown in Table 4.

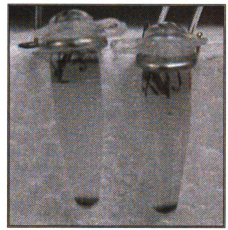

Figure 7. Protein A gels after the cells were reacted with anti-DEA 1.X DEA1.X positive cells are in the tube on the left, and DEA 1.X negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.X and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

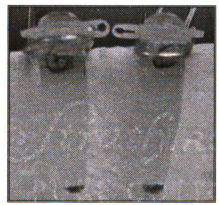

Figure 8. Protein G gels after the cells were reacted with anti-DEA 1.X DEA1.X positive cells are in the tube on the left, and DEA 1.X negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.X and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

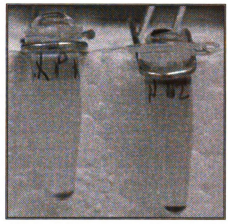

Figure 9. Protein L gels after the cells were reacted with anti-DEA 1.X DEA1.X positive cells are in the tube on the left, and DEA 1.X negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.X and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

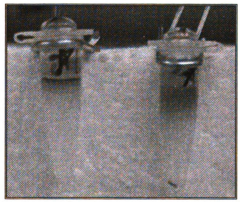

Figure 10. ACG gels after the cells were reacted with anti-DEA 1.X DEA1.X positive cells are in the tube on the left, and DEA 1.X negative cells are on the right. Both sets of cells were incubated with anti-DEA 1.X and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Table 4: Results for screening effectiveness for anti-DEA 1.X sensitized cells Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). The term "positive" refers to the tube with the cells containing DEA 1.X antigen. "Negative" refers to the tube with cells that do not contain the antigen.

	run #1	run #2
Protein		
A		
positive	0	0
negative	0	0
G		
positive	0	0
negative	0	0
L		
positive	0	0
negative	0	0
ACG		
positive	4+	4+
negative	3+	3+
Test tube		
positive	3+	3+
negative	0	0
positive w/Coombs	4+	4+
negative w/Coombs	0	0
Plain Gel		
positive	3+	
negative	0	

Screening the gels using anti-DEA 3,5 sensitized erythrocytes

Examples of gels A, G, L, and ACG are shown in Figure 11, Figure 12, Figure 13, and Figure 14 respectively. The results of screening gels A, G, L, ACG, control, and test tube using anti-DEA 3,5 sensitized erythrocytes are shown in Table 5.

Figure 11. Protein A gels after the cells were reacted with anti-DEA 3,5 DEA 3 positive cells are in the tube on the left, and DEA 3 negative cells are on the right. Both sets of cells were incubated with anti-DEA 3,5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

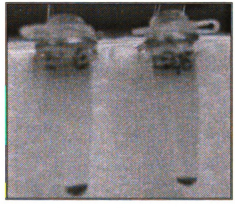

Figure 12. Protein G gels after the cells were reacted with anti-DEA 3,5 DEA 3 positive cells are in the tube on the left, and DEA 3 negative cells are on the right. Both sets of cells were incubated with anti-DEA 3,5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

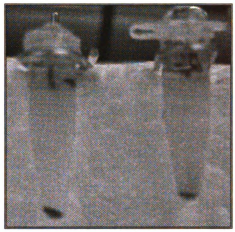

Figure 13. Protein L gels after the cells were reacted with anti-DEA 3,5 DEA 3 positive cells are in the tube on the left, and DEA 3 negative cells are on the right. Both sets of cells were incubated with anti-DEA 3,5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

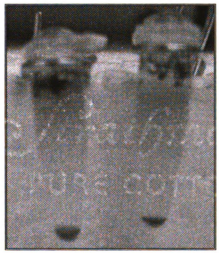

Figure 14. ACG gels after the cells were reacted with anti-DEA 3,5 DEA 3 positive cells are in the tube on the left, and DEA 3 negative cells are on the right. Both sets of cells were incubated with anti-DEA 3,5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Table 5. Results for screening effectiveness for anti-DEA 3,5 sensitized cells Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). The term "positive" refers to the tube with the cells containing DEA 3 antigen. "Negative" refers to the tube with cells that do not contain the antigen.

	run #1	run #2	run #3	4 ° C
Protein				
A				
positive	0	0	0	0
negative	0	0	0	0
G				
positive	0	0	0	0
negative	0	0	0	0
L				
positive	0	0	0	0
negative	0	0	0	0
ACG				
positive	0	0	0	0
negative	0	0	0	0
Testtube				
positive	2+	2+	2+	2+
negative	0	0	0	0
Plain gel				
positive	0			
negative	0			

Screening the gels using anti-DEA 4 sensitized erythrocytes

Examples of gels A, G, L, and ACG are shown in Figure 15, Figure 16, Figure 17, and Figure 18 respectively. The results of screening gels A, G, L, ACG, control, and test tube using anti-DEA 4 sensitized erythrocytes are shown in Table 6.

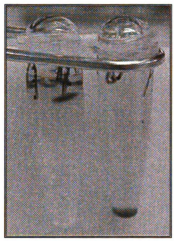

Figure 15. Protein A gels after the cells were reacted with anti-DEA 4 DEA 4 positive cells are in the tube on the left, and DEA 4 negative cells are on the right. Both sets of cells were incubated with anti-DEA 4 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

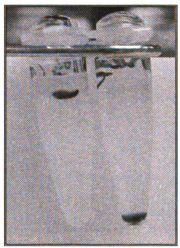

Figure 16. Protein G gels after the cells were reacted with anti-DEA 4 DEA 4 positive cells are in the tube on the left, and DEA 4 negative cells are on the right. Both sets of cells were incubated with anti-DEA 4 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

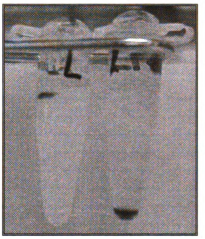

Figure 17. Protein L gels after the cells were reacted with anti-DEA 4 DEA 4 positive cells are in the tube on the left, and DEA 4 negative cells are on the right. Both sets of cells were incubated with anti-DEA 4 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Figure 18. ACG gels after the cells were reacted with anti-DEA 4 DEA 4 positive cells are in the tube on the left, and DEA 4 negative cells are on the right. Both sets of cells were incubated with anti-DEA 4 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Table 6. Results for screening effectiveness for anti-DEA 4 sensitized cells Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). The term "positive" refers to the tube with the cells containing DEA 4 antigen. "Negative" refers to the tube with cells that do not contain the antigen.

	run #1	run #2	run #3
Protein			
A			
positive	4+	4+	4+
negative	0	0	0
G			
positive	4+	4+	4+
negative	0	0	0
L			
positive	4+	4+	4+
negative	0	0	0
ACG			
positive	4+	4+	4+
negative	0	0	0
Test tube			
positive	4+	4+	4+
negative	0	0	0
Plain Gel			
positive	4+		
negative	0		

Screening the gels using anti-DEA 5 sensitized erythrocytes

Examples of gels A, G, L, and ACG are shown in Figure 19, Figure 20, Figure 21, and Figure 22 respectively. The results of screening gels A, G, L, ACG, control, and test tube using anti-DEA 5 sensitized erythrocytes are shown in Table 7.

Figure 19. Protein A gels after the cells were reacted with anti-DEA 5 DEA 5 positive cells are in the tube on the left, and DEA 5 negative cells are on the right. Both sets of cells were incubated with anti-DEA 5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

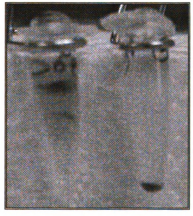

Figure 20. Protein G gels after the cells were reacted with anti-DEA 5 DEA 5 positive cells are in the tube on the left, and DEA 5 negative cells are on the right. Both sets of cells were incubated with anti-DEA 5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Figure 21. Protein L gels after the cells were reacted with anti-DEA 5
DEA 5 positive cells are in the tube on the left, and DEA 5 negative cells are on the right. Both sets of cells were incubated with anti-DEA 5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

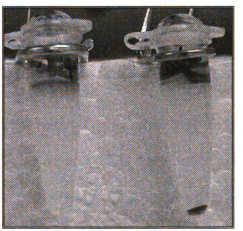

Figure 22. ACG gels after the cells were reacted with anti-DEA 5
DEA 5 positive cells are in the tube on the left, and DEA 5 negative cells are on the right. Both sets of cells were incubated with anti-DEA 5 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Table 7. Results for screening effectiveness for anti-DEA 5 sensitized cells Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). The term "positive" refers to the tube with the cells containing DEA 5 antigen. "Negative" refers to the tube with cells that do not contain the antigen.

	run #1	run #2	run#3
Protein			
A			
positive	3+	3+	3+
negative	0	0	0
G			
positive	2-3+	3+	2+
negative	0	0	0
L			
positive	1-2+	2+	1-2+
negative	0	0	0
ACG			
positive	4+	4+	4+
negative	0	0	0
Test tube			
positive	2+	3+	3+
negative	0	0	0
Plain Gel			
positive	2+		
negative	0		

Screening the gels using anti-DEA 7 sensitized erythrocytes

Examples of gels A, G, L, and ACG are shown in Figure 23, Figure 24, Figure 25, and Figure 26 respectively. The results of screening gels A, G, L, ACG, control, and test tube using anti-DEA 7 sensitized erythrocytes are shown in Table 8. Both the DTT treated and the PBS sera demonstrated a 3+ reaction. The negative DTT control was negative.

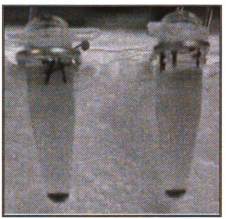

Figure 23. Protein A gels after the cells were reacted with anti-DEA 7 DEA 7 positive cells are in the tube on the left, and DEA 7 negative cells are on the right. Both sets of cells were incubated with anti-DEA 7 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

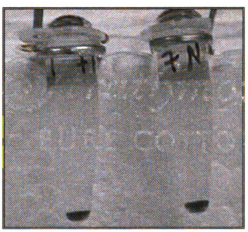

Figure 24. Protein G gels after the cells were reacted with anti-DEA 7 DEA 7 positive cells are in the tube on the left, and DEA 7 negative cells are on the right. Both sets of cells were incubated with anti-DEA 7 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

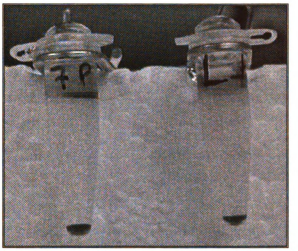

Figure 25. Protein L gels after the cells were reacted with anti-DEA 7 DEA 7 positive cells are in the tube on the left, and DEA 7 negative cells are on the right. Both sets of cells were incubated with anti-DEA 7 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

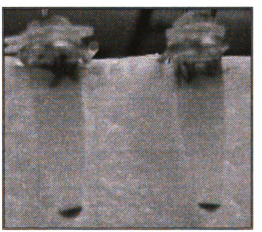

Figure 26. ACG gels after the cells were reacted with anti-DEA 7 DEA 7 positive cells are in the tube on the left, and DEA 7 negative cells are on the right. Both sets of cells were incubated with anti-DEA 7 and washed three times before being transferred to the gels and centrifuged at 1200 g for 10 min.

Figure 27: Protein A gel with anti-DEA 7 and anti-DEA 4 sensitized cells The initial test, with anti-DEA 7 sensitized cells, is responsible for the cell population at the bottom, and later test, with anti-DEA 4 sensitized cells, is responsible for the cell population at the top.

Table 8. Results for screening effectiveness for anti-DEA 7 sensitized cells Results scored on a scale of 0 (no agglutination) to 4+ (complete agglutination). The term "positive" refers to the tube with the cells containing DEA 7 antigen. "Negative" refers to the tube with cells that do not contain the antigen.

	run #1	run #2	run #3	4 °C
Protein				
A				
positive	0	0	0	0
negative	0	0	0	0
G				
positive	0	0	0	0
negative	0	0	0	0
L				
positive	0	0	0	0
negative	0	0	0	0
ACG				
positive	0	0	0	0
negative	0	0	0	0
Test tube				
positive	3+	3+	3+	2+
negative	0	0	0	0
Plain Gel				
positive	0			
negative	0			

Discussion

The conditions chosen for the microcentrifuge, 1200 g for 10 min, were chosen for two reasons. First, the positive reaction appeared positive and the negative appeared negative. The second reason was that it was only a 10 min centrifugation as opposed to 1050 g for 15 min, which also showed a clear positive and negative.

The ACG gel demonstrated the strongest reaction to erythrocytes sensitized to anti-DEA 1.1 followed by the control gel which was stronger than the test tube. Gels A and L reacted, but were weaker than the control gel. Gel G had no visible reaction to the sensitized cells (Table 3).

Anti-DEA 1.X sensitized cells reacted in the ACG positive and negative gels. The other gels were ineffective (Table 4). The unexpected positive in the ACG tube with DEA 1.X antigen negative erythrocytes is not due to the reaction of dog A's erythrocytes alone, since dog A cells unreacted with anti-sera resulted in a negative reaction when centrifuged through the gel. There must be a reaction occurring due to the sera, or that particular sera in combination with dog A's cells. If more sera were available, this could be tested by using cells from another dog negative for DEA 1. The gel itself reacted as expected in screens performed before and after this one; therefore it is not likely that the gel was compromised.

None of the gels displayed any reaction to anti-DEA 3,5 sensitized cells. The test tube showed a moderately positive reaction (Table 5). After performing one run where the equipment and the reagents were all at 4 °C, the reactions

remained unchanged. This run was performed with the intent of attempting to enhance the reaction, although anti-DEA 3,5 reaction is known to occur at room temperature as well.

All positive gels maintained a strong positive reaction to anti-DEA 4 sensitized cells. The positive test tube was also very strong (Table 6). The immunoglobulin binding proteins present in the gels were not necessary to observe a strong reaction.

The ACG gel showed the strongest reaction to anti-DEA 5 sensitized cells. Protein G and control gels showed moderate reactivity similar to the test tube method. Gel L was slightly weaker than the test tube (Table 7). The reaction strength displayed in the ACG gel suggest that not only sieving properties are responsible for the strength, but the ACG is likely acting as an immunoglobulin binding protein against the immunoglobulins present on the sensitized cells.

The test tube positive DEA 7 reaction was a consistent moderately strong reaction, and the negative tubes were negative. In contrast, all gels were unreactive to erythrocytes sensitized with anti-DEA 7 (Table 8). The protein A gel that was previously run with anti-DEA 7 sensitized cells and rerun with anti-DEA 4 sensitized cells, was used to test the function of the centrifuge and the gels. This resulted in a strong positive reaction for anti-DEA 4 sensitized erythrocytes, indicating that the gel was functional. Performing an entire run at 4 °C, including equipment and reagents, was done in an attempt to enhance any cold reaction that may have been missed with the initial method. Enhancement did not occur. The addition of DTT would have destroyed the ability of IgM to

agglutinate or activate complement.[34] Immunoglobulin M is not likely to be fully responsible for the anti-DEA 7 activity since DTT treatment did not alter the reactivity. The lack of positive results in the gels may be due to the fact that DEA 7 antigen may be adsorbed onto the cell similar to the human Lewis blood group system,[24] and therefore, may be removed from the cells during the gel testing procedure.

It remains unclear why any of the protein gels would appear negative while the plain gel was positive, but this occurred for only the anti-DEA 1.1 serum and only comparing the plain gel to protein A, G, and L gels (not the ACG gel). This suggests either a difference between the gels themselves or differences resulting from gel handling and processing. Gel particles were sold as being of the same size, and the method for attaching the proteins was the same for each. However, the plain and ACG gels were processed in-house, and the other gels were processed before purchase. Thus, differences in processing are possible. It is also likely that the gels themselves came from different lots for the different proteins. Perhaps there exists delicate balance between the narrowness of the tube, which is important for protein interaction with immunoglobulins, and the centrifuge conditions necessary for a sieving effect. A gel with a smaller range in gel particle size may provide more consistent sieving properties.

The grading of the gel was more difficult than the human method for the weaker reactions. This may be due to the shape of the PCR tube. Cells often stay to the sides of the tube in the conical portion; therefore they may not be experiencing the sieving effect in this portion of the tube. The human gel cards

have a long narrow column with parallel sides, while a PCR tube or microcentrifuge tube has a shorter column and a conical shape with tapering sides of the tube.

Since most DEA reactions were visible in the control gel and the ACG gel, they are likely to be the best matrices to use for immunochromatographic methods of detecting immunohematologic reactions in dogs. The control gel is the more cost effective choice. The reactions are less strong than in the ACG gel; however, they are easily discernable from a negative. The strength of the reaction in the ACG gel is likely greater due to the protein (ACG)-IgG interaction.

Future prospective

Currently, a plain gel matrix seems to be the best gel option. ACG gel is also very effective. Though more expensive, its reactions are sometimes stronger than the plain gel. If a cost effective source of ACGs, particularly a mixture of monoclonal antibodies, is developed, an ACG gel for compatibility testing may prove to be more useful. To apply the immunoglobulin binding protein gel to DEA typing, a monoclonal antibody for each significant DEA type, that is also recognized by an immunoglobulin binding protein like protein A (the most cost effective protein in this study), could theoretically provide consistent, reliable results. If cost-effective monoclonal antibodies are developed, it may be less expensive to create an immunochromatographic gel because less serum, compared to the test tube method, would be necessary to achieve a positive result.

BIBLIOGRAPHY

1. *Technical Manual 14th ed.*, American Association of Blood Banks, Bethesda, MD 2002; Chapter 18: Pretransfusion Testing: 379-392.

2. Harrell KA, Kristensen AT. "Canine Transfusion Reactions and Their Management." *The Veterinary Clinics of North America. Small Animal Practice* 1995; 25(6): 1333-1364.

3. *Technical Manual. 14th ed.*, American Association of Blood Banks, Bethesda, MD 2002; Chapter 27: Noninfectious Complications of Blood Transfusion: 585-612.

4. Cotter SM, "Comparative Transfusion Medicine", Advances in Veterinary Science and Comparative Medicine 1991; 36: 2-6.

5. Swisher SC, Young LE, "The Blood Grouping Systems of Dogs." *Physiology Review* 1961; 41: 495-520.

6. *Technical Manual. 14th ed.*, American Association of Blood Banks, Bethesda, MD 2002; Chapter 12: Red Cell Antigen-antibody Reaction and Their Detection: 253-269.

7. *Technical Manual 14th ed.*, American Association of Blood Banks, Bethesda, MD 2002; Chapter 11: Immunology: 225-252.

8. "Gel Test Advantages." Microtyping Systems Inc. Jan 7, 2003, http://www.idmts.com/gel_test.html.

9. Malyska H, Weiland D, "The Gel Test." *Laboratory Medicine* 1994;25:81-5.

10. Lapierre V, Rigal D, Adam J, et al., "The Gel Test: A New Way to Detect Red Cell Antigen-antibody Reactions." *Transfusion* 1990; 30: 109-113.

11. Duguid JKM, Bromilow IM, "New Technology in Hospital Blood Banking." *Journal of Clinical Pathology* 1993; 46(7): 585-8.

12. Hara Y, Ejima H, Aoki S, et al, "Preparation of Monoclonal Antibodies Against Dog Erythrocyte Antigen D1 (DEA-3)." *Journal of Veterinary Medical Science* 1991; 53(6): 1105-1107.

13. Andrews GA, Chavey PS, Smith JE, "Production, Characterization, and Applications of a Murine Monoclonal Antibody to Dog Erythrocyte Antigen 1.1." *Journal of the American Veterinary Medical Association* 1992; 10: 1549-1552.

14. Giger U, Stieger K, Airasmaa H, "Initial Comparison of Various Canine Blood Typing Methods." an abstract in *21st ACVIM Proceedings* 2003.

15. Vriesendorp HM, Albert ED, Templeton JW, et al., "Joint Report of the Second International Workshop on Canine Immunogenetics." *Transplantation Proceedings* 1976; 8(2): 289-311.

16. Hale AS, "Canine Blood Groups and Their Importance in Veterinary Transfusion Medicine." The*Veterinary Clinics of North America. Small Animal Practice* 1995; 25: 1323-1332.

17. Swisher SN, Young LE, Trabold N, "In vitro and in vivo Studies of the Behavior of Canine Erythrocyte-isoantibody Systems." *Annals of the New York Academy of Sciences* 1962; 9715-9725.

18. Young LE, O'Brien WA, Swisher SN, Miller G, Yuille CL, "Blood Groups in Dogs-Their Significance to the Veterinarian." *American Journal of Veterinary Research* 1952; 13: 207-213.

19. Andrews GA, *Schalm's Veterinary Hematology 5th ed.*, Baltimore, MD 2000; Chapter 112: Red Blood Cell Antigens and Blood Groups in the Dog and Cat: 767-773.

20. Symons M, Bell K, "Expansion of the Canine A Blood Group System." *Animal Genetics* 1991; 22: 227-235.

21. Corato A, Mazza G, Hale AS, Barker RN, Day MJ, "Biochemical Characterization of Canine Blood Group Antigens: Immunoprecipitation of DEA 1.2, 4, and 7 and Identification of a Dog Erythrocyte Membrane Antigen Homologous to Human Rhesus." *Veterinary Immunology and Immunopathology* 1997; 59: 213-223.

22. Juneja RK, Gerlach JA, Hale AS, *The Genetics of the Dog*, CABI Publishing, NY 2001; Biochemical Genetics and Blood Groups: Blood Groups: 126-137

23. Bull RW, Vriesendorp HM, Zweibaum A, Swisher SN, "The Inapplicability of CEA-7 as a Canine Bone Marrow Transplantation Marker." *Transplantation Proceedings* 1975; 7(4): 575-577.

24. "MTS Anti-IgG Card" (insert), Microtyping Systems, Inc., Pompano Beach, Florida. 1997, Diagram 1.

25. Dittmar K, Procter JL, Cipolone K, Njoroge JM, Miller J, Stroncek DF, "Comparison of DATs using Traditional Tube Agglutination to Gel Column and Affinity Column Procedures." *Transfusion* 2001; 41(10): 1258-1262.

26. Cate JC, Reilly N, "Evaluation and Implementation of the Gel Test for Indirect Antiglobulin Testing in a Community Hospital Laboratory." *Archives of Pathology and Laboratory Medicine* 1999; 123: 693-697.

27. Sjoquist J, Meloun B, Hjelm H, "Protein A Isolated from Staphylococcus aureus after Digestion with Lysostaphin." *European Journal of Biochemistry* 1972; 29: 572-578.

28. Bjorck I, Kronvall G, "Purification and Some Properties of Streptococcal Protein G, a Novel IgG-binding Agent." *Journal of Immunology* 1984; 133: 969-974.

29. Peng Z, Simons R, Becker AB, "Differential Binding Properties of Protein A and Protein G for Dog Immunoglobulins." *Journal of Immunological Methods* 1991; 145: 255-258.

30. Akerstrom B, Bjorck L, "Protein L: An Immunoglobulin Light Chain-binding Bacterial Protein." *Journal of Biological Chemistry* 1989; 264: 19740-19746.

31. Housden NG, Harrison S, Roberts SE, Beckingham JA, Graille M, Stura E, Gore MG, "Immunoglobulin-binding Domains: Protein L from Peptostreptococcus magnus." *Biochemical Society Transactions* 2003; 31(3): 716-718.

32. DeChateau M, Nilson BH, Erntell M, Myhre E, Magnusson CG, Akerstrom B, Bjork L, "On the Interaction Between Protein L and Immunoglobulins of Various Mammalian Species." *Scandinavian Journal of Immunology* 1993; 37(4): 399-405.

33. Scott MA, Davis JM, Schwartz KA, "Staphylococcal Protein A Binding to Canine IgG and IgM." *Veterinary Immunology and Immunopathology* 1997; 59: 205-212.

34. *Technical Manual 14th ed.*, American Association of Blood Banks, Bethesda, MD 2002; Method 3.9: Use of Sulfhydryl Reagents to Distinguish IgM from IgG Antibodies: 700-701.