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# Alloimmunization of a dog erythrocyte antigen 1− dog transfused with weakly dog erythrocyte antigen 1+ blood

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## Abstract

**Background:** Acute hemolytic transfusion reactions because of dog erythrocyte antigen (DEA) 1 sensitization after mismatched transfusions are serious complications. Dog erythrocyte antigen 1 expression varies from negative to weakly to strongly positive.

**Objectives:** To assess alloimmunization after transfusion of weakly DEA 1+ blood to a DEA 1− dog.

**Animals:** One DEA 1− recipient and 1 weakly DEA 1+ donor, and 106 control dogs.

**Methods:** Long-term follow-up study. Matched for DEA 3, 4, 5, and 7, Dal, and Kai 1 and 2, weakly DEA 1+ donor packed red blood cells (RBCs) were transfused 3 times (0.45 mL/kg at Day 0, 16, and 37) to a DEA 1− recipient. Alloantibodies against RBCs from donor and 106 controls were determined in recipient's plasma samples using a commercial antiglobulin-enhanced immunochromatographic strip and gel tube crossmatches. Alloantibody titers were determined.

**Results:** The DEA 1− recipient was sensitized after 16 days to ≥1657 days after transfusion to weakly DEA 1+ and otherwise matched RBCs. Strong to moderate crossmatch incompatibilities were observed between recipient's plasma and all 61 DEA 1+ crossmatched controls. Moderate to weak incompatibilities were also observed to DEA 1− controls. Anti-DEA 1 and other alloantibodies were detected over the 4.5 year observation period.

**Conclusions and Clinical Importance:** Blood from a weakly DEA 1+ donor induces a strong and durable alloimmunization in a DEA 1− recipient dog. Additional alloantibodies developed against yet to be defined RBC antigens. Those results support the recommendation of typing dogs against DEA 1, considering weakly DEA 1+ as immunogenic, and crossmatching all previously transfused dogs.

KEYWORDS

alloantibodies, blood compatibility, canine, dog erythrocyte antigen, hemolytic transfusion reaction

**Abbreviations:** ACD, acid citrate dextrose; AGC, antiglobulin-enhanced gel column; AIC, antiglobulin-enhanced immunochromatographic crossmatch; Control, control dog; DEA, dog erythrocyte antigen; Donor, donor dog; Ig, immunoglobulin; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; RBC(s), red blood cell(s); Recipient, recipient dog.

## 1 | INTRODUCTION

More than a dozen blood groups have been reported in dogs and some have been classified as dog erythrocyte antigens (DEAs), but polyclonal (antisera) and/or monoclonal alloantibodies (typing reagents) are only available for some.[1-4] Based upon extensive clinical experience, we found that dogs do not have any clinically important naturally occurring alloantibodies,[5,6] albeit anti-DEA 7 has been described.[7,8] Experimental investigations and a few case studies revealed that dogs can be sensitized by mismatched blood transfusions and they develop acute hemolytic transfusion reactions, if transfused again >4 days from the same or other donors.[9,10]

The DEA 1 is considered clinically the most important blood group in dogs because of its strong antigenicity and nearly equal distribution of DEA 1+ and DEA 1− dogs among many breeds worldwide.[11] Based upon our recent surveys using quantitative immunochromatographic strip methods, most dogs are either DEA 1− or moderately to strongly DEA 1+.[12,13] However, few dogs are only weakly DEA 1+, and these dogs might be mistyped as DEA 1−. Although DEA 1 mismatched transfusions have been reported to cause anti-DEA 1 alloantibodies and acute hemolytic transfusion reactions, the antigenicity of weakly DEA 1+ blood transfused to a DEA 1− dog is unknown.

In this study, we investigated the course of alloimmunization of a DEA 1− recipient dog by transfusing weakly DEA 1+ blood and an otherwise matched donor and recipient pair utilizing antiglobulin-enhanced immunochromatographic strip and gel tube crossmatch kits.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals, immunization, and blood sample collection and preparation

A 6-year-old healthy DEA 1− Border Collie (Recipient, 22 kg body weight), a 2-year-old weakly DEA 1+ Appenzell Mountain blood donor dog (Donor, weighing 25 kg), and 106 healthy control blood donors (Controls, mixed breed and purebred dogs), owned by veterinary students and hospital staff, were used in this study over a 4.5-year period from 2013 to 2018. All dogs were considered healthy at time of blood collections based on annual physical examination, CBCs, and infectious disease screen. The study was approved by the Institutional Animal Care and Use and Ethics Committee of VetAgro Sup (#1267), and owner consent was obtained before Recipient and Donor enrollment into the study and blood collection.

### 2.2 | Alloimmunization schedule

Based upon extended blood group results, a Recipient–Donor pair was selected. During October and November 2013, 40 mL of Donor blood was collected into a 60-mL syringe containing acid citrate dextrose (ACD) solution from Blood Pack Units (Macopharma, Tourcoing, France) on 3 separate occasions. The anticoagulated blood was immediately centrifuged, the plasma was separated and frozen, and the packed red blood cells (RBCs) were washed 3 times in sterile phosphate-buffered saline (PBS).[14] In the intensive care unit under close medical supervision, 10 mL (0.45 mL/kg) of freshly washed Donor packed RBCs were transfused to Recipient via saphenous vein on Days 0, 16, and 37 (Figure 1). Temperature, pulse, respiration, and attitude of the Recipient were regularly assessed during and after transfusion, although signs of hemolysis or hyperbilirubinemia were not followed specifically.

### 2.3 | Diagnostic blood sample collections

Diagnostic blood samples were collected from the jugular or saphenous veins from all dogs into 6-mL tubes containing ACD (ACD Solution B, Becton Dickinson, Plymouth, the United Kingdom). These samples were chilled and within 1 week, they were either directly used or at that time washed 3 times in PBS for blood typing. The ACD tubes with the remaining blood were centrifuged (at 1300*g* for 10 minutes) on the collection day to separate plasma and RBCs for crossmatch testing and alloantibody titer determinations. The RBCs were stored at 4°C for <10 days, whereas plasma samples were frozen at −20°C until used for crossmatch tests.

Blood samples were drawn from Donor and Recipient before and after transfusions on Days 0 (first transfusion), 4, 13, 16 (second transfusion), 22, 37 (third transfusion), 48, 106, 891 (2.4 years), and 1657 (4.5 years) and centrifuged to obtain plasma, which was frozen at −20°C. Blood samples from Control donor dogs were obtained around the days of Recipient–Donor pair blood collection on Days 48 to 1657 after first transfusion and were used as "Panel RBCs" against the Recipient's plasma samples (Figure 1).

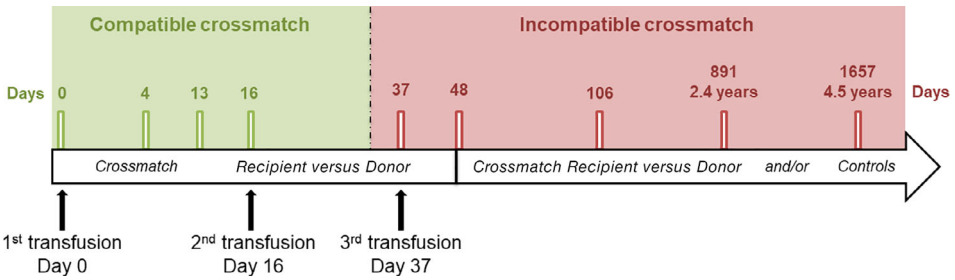


**FIGURE 1** Timeline of compatibility testing between Recipient and Donor or Controls throughout immunization and follow-up period. The time line of the study took place from Day 0 (October 2013) to Day 1657 (April 2018). Recipient plasma samples from Days 106 and 891 were frozen to be crossmatched later with Control red blood cells (RBCs)

### 2.4 | Typing for DEA 1, DEA 3, DEA 4, DEA 5, DEA 7, Kai 1, Kai 2, and Dal

Typing for DEA 1 was performed in every blood sample collected by a semi- and a quantitative method. An in-clinic immunochromatographic strip kit (Alvedia, Limonest, France) and a laboratory flow cytometric technique utilizing the same monoclonal murine anti-DEA 1 antibody were used to type for DEA 1 as per manufacturer instructions or as previously described.[5,12] For the Recipient, Donor, and 1 of the Controls, typing for DEA 3, DEA 5, and Dal using polyclonal antibodies and Kai 1 and Kai 2[3,4,15,16] using monoclonal antibodies was performed at PennGen Laboratories (School of Veterinary Medicine, University of Pennsylvania, Philadelphia) and typing for DEA 4 and DEA 7[3] using polyclonal antibodies was done at Animal Blood Resources International (Stockbridge, Michigan), as previously described.[17]

### 2.5 | Two crossmatch tests and alloantibody titer determinations

Major and minor crossmatch tests were performed before and after the first transfusion (Days 0-1657) between Recipient and Donor as well as with Panel RBCs from Day 48 until Day 1657. The degrees of incompatibilities were assessed overtime semiquantitatively between RBCs from the same 11 (6 DEA 1+, 5 DEA 1–) of the 106 Controls and Recipient's plasma from Days 106, 891, and 1657. A commercial canine-specific antiglobulin-enhanced immunochromatographic crossmatch (AIC) strip kit[5] and an antiglobulin-enhanced gel column (AGC) test kit technique[18] were used, and results were interpreted according to the manufacturer's instructions (Alvedia) and as previously described.[5]

### 2.6 | Major crossmatch between Recipient's plasma and Controls RBCs using AGC

Following manufacturer's instructions,[18] 50 μL of 1% packed Donor RBCs in a low ionic strength solution (Bio-Rad, DiaMed GmbH, Cressier, Switzerland) were added to 25 μL of Recipient plasma in a 3-mL polystyrene test tube, briefly mixed, and incubated at 22°C for 10 minutes. After incubation, 40 μL of the RBC suspensions were gently added on top of the gel microtube containing a canine antiglobulin reagent that reacts to canine immunoglobulin (Ig) G, IgM, and complement. The gel microtubes were then centrifuged at 200*g* for 10 minutes, and the location of the migrated RBCs was recorded. In the absence of agglutination, the RBC passed through the gel to the bottom, which was scored as "compatible," whereas agglutination on the top of or within the gel was considered "incompatible." Autocontrols (with RBCs and plasma from the same dog) were also included for all crossmatch tests performed.

For each crossmatch test, the strength of the agglutination reaction was recorded as follows: 0 (negative), all RBCs were at the bottom of the tube; 1+ (positive), few RBCs' agglutinates were dispersed in the gel, but most of the RBCs were at the bottom of the tube; 2+ (positive), all RBCs' agglutinates were dispersed in the gel; 3+ (positive), some RBCs' agglutinates were dispersed in the upper part of the gel, most of the RBCs form a red line on the surface of the gel; and 4+ (positive), all RBCs were agglutinated and form a red line on the surface of the gel.[2,5,18,19]

### 2.7 | Alloantibody titration in recipient's plasma

Titer measurements for alloantibodies in Recipient's plasma from Day 106 to Day 1657 were obtained with 2-fold serial dilutions of Recipient's plasma in PBS mixed with only Donor RBCs utilizing the AGC test as described above.

### 2.8 | Anti-DEA 1 alloantibodies specificity in adsorption study

One milliliter of Recipient's plasma from Day 106 was separately incubated overnight at 37°C under 2 conditions with 1 mL of either strongly DEA 1+ Control or a DEA 1– Control washed RBCs. Both suspensions were then centrifuged for 10 minutes at 2000*g*. The resulting supernatants were again incubated as described above.[13,20] These supernatants from adsorbed plasma were crossmatched using only the AIC technique against the same DEA 1+ Control RBCs.

## 3 | RESULTS

### 3.1 | Blood typing

The categorical and the semiquantitative and quantitative DEA 1 blood typing results were completely concordant between the flow cytometric and immunochromatographic strip typing techniques for Recipient, Donor, and all 106 Controls (Figure 2 and Table 1). The Recipient and Donor were DEA 1 typed repeatedly and consistently showed the same results. The Donor RBCs expressed weakly DEA 1+ (1+ band strength, MFI = 28, Figure 2), whereas the Recipient was DEA 1– (0 band; MFI = 3, Figure 2) and matched for all other tested blood groups, thereby limiting sensitizing outside DEA 1. Extended blood typing for the Recipient, Donor, and 1 of the Controls for DEA 3, 4, 5, and 7, Dal, and Kai 1 and 2 performed before and twice after transfusion revealed consistently a perfect match for tested blood groups except the additional Control that tested DEA 7+ as opposed to Donor and Recipient (Table 1).

### 3.2 | Pre- and postalloimmunization compatibility testing

#### 3.2.1 | Autocontrol and minor crossmatch test results

There was no autoagglutination observed in any autocontrol tests when mixing plasma and washed RBCs from Recipient, Donor, and all Controls studied by either crossmatch method. Neither was any agglutination noted when crossmatching Recipient's RBCs against plasma from Donor nor it was noted in 106 Controls tested (compatible minor crossmatch) at any time points.

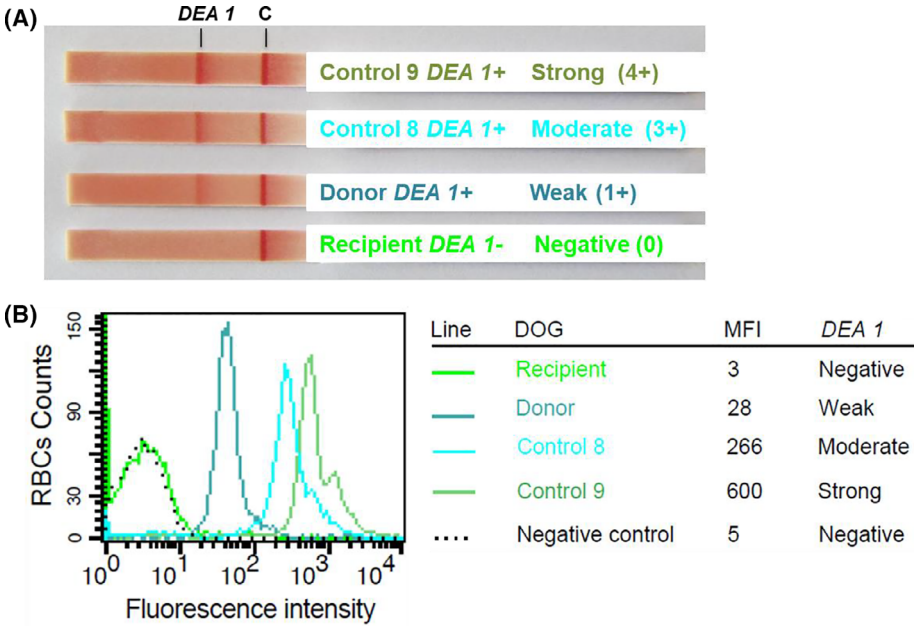


**FIGURE 2** Expression of dog erythrocyte antigen (DEA) 1 in Recipient, Donor, and 2 Controls assessed by A, immunochromatographic and B, flow cytometric techniques. The DEA 1 immunochromatographic typing strip has 1 line with a control antibody (labeled "C") present on all canine red blood cells (RBCs) (thus, a red band at "C" indicates that the test is valid) and any band intensity at the "DEA 1" position is considered positive (graded 1+ to 4+). Dog erythrocyte antigen 1 expression was classified as negative (mean fluorescence intensity [MFI] <10), weakly positive (10 ≤ MFI < 100), moderately positive (100 ≤ MFI < 300), and strongly positive (MFI ≥ 300) by flow cytometric analysis

**TABLE 1** Extended blood type of Recipient, Donor, and 1 Control

| | DEA 1 | DEA 3 | DEA 4 | DEA 5 | DEA 7 | Dal | Kai 1 | Kai 2 |
|---|---|---|---|---|---|---|---|---|
| Recipient | – | – | + | – | – | + | + | – |
| Donor | + | – | + | – | – | + | + | – |
| Control | – | – | + | – | + | + | + | – |

*Note*: Results were recorded as follows: –, negative; +, positive.

### 3.2.2 | Major crossmatch between Recipient and Donor before, during, and after alloimmunization

A major crossmatch was performed from before and throughout the entire observation period of 4.5 years after transfusion between Recipient and Donor. The major crossmatch by both tests (AIC and AGC) between Recipient's plasma and Donor RBCs were compatible before transfusion. Moreover, the major crossmatch remained compatible when tested at Days 4, 13, and 16 from the first transfusion (Day 0). However, as of Day 37—the next time a major crossmatch was performed—there was strong incompatibility (4+) observed which remained until the end of the study at 4.5 years. The AIC and AGC techniques revealed similar incompatibility binding and agglutination reactions, respectively, between Recipient and Donor after alloimmunization with decreasing crossmatch incompatibilities (3+) on Days 891 and 1657 (Figure 3 and Table 2).

### 3.2.3 | Alloantibody titration in recipient's plasma

The Recipient's plasma alloantibody titers measured from Day 106 to Day 1657 were strong against the Donor RBCs and declined over time. Strong incompatibilities (4+) were observed up to Donor plasma dilutions of 1:256 until Day 106, whereas Recipient plasma samples from Days 891 and 1657 showed strong agglutination reactions only until plasma dilutions of 1:8. A negative agglutination reaction was reached at plasma titers of 1:4096 on Day 106, 1:1024 on Day 891, and 1:256 on Day 1657 (Figure 4).

### 3.2.4 | Major crossmatch between Recipient's plasma and Controls RBCs using AGC

Moderate-to-strong major crossmatch incompatibilities were observed between Recipient's plasma and all 11 Controls with DEA 1+ RBCs tested from Day 48 to Day 1657. On Day 48, Recipient's plasma was also incompatible against all 4 DEA 1– Controls tested (Table 2). On Days 106 and 891, the plasma from all 50 tested DEA 1+ RBCs showed incompatibilities with the Recipient's plasma from both dates. In addition, 24 (63%) and 9 (24%) of the 38 tested DEA 1– RBCs were also showing incompatibilities with Recipient's plasma from Days 106 and 891, respectively. Finally, on Day 1657, all 6 (100%) DEA 1+ RBCs tested and 3 (60%) DEA 1– RBCs of the 5 tested were incompatible with Recipient's plasma (Table 2).

The degrees of incompatibilities were assessed overtime semiquantitatively between RBCs from the same 11 (6 DEA 1+, 5 DEA 1–) of the 106 Controls and Recipient's plasma from Days 106, 891, and 1657. The incompatibilities with DEA 1+ RBCs from all 6 Controls remained fairly stable at 2+ to 4+ throughout. Furthermore, 3 of the 5 DEA 1– RBCs also showed strong incompatibilities (3+) with

Recipient's plasma on Day 106. Interestingly, further follow-up revealed persistently strong incompatibility with 2 DEA 1– Controls and decreasing incompatibility with 1 Control (1+), whereas 1 Control became again compatible (Figure 5 and Table 2).

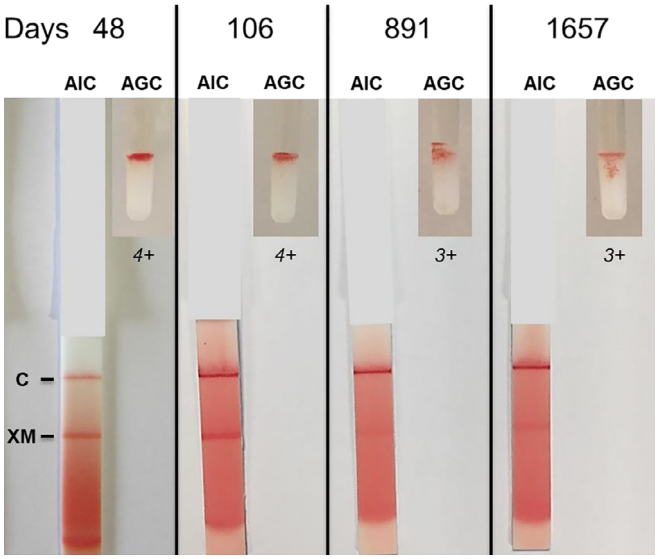


**FIGURE 3** Major crossmatch incompatibilities between Recipient and Donor after transfusion of weakly DEA 1+ blood to a DEA 1– dog using an antiglobulin-enhanced immunochromatographic strip crossmatch (AIC) and an antiglobulin-enhanced gel tube crossmatch (AGC) technique. Antiglobulin-enhanced immunochromatographic crossmatch test has a band with a control antibody on the strip (labeled "C") specific to all canine red blood cells (RBCs) (thus, a red band at "C" indicates that the test is valid). Any band intensity at "XM" is considered positive (graded 1+ to 4+) indicating alloantibodies are present against IgG, IgM, and/or C3 on the RBCs surface and thus indicating incompatibility. Agglutination reactions in AGC tests are recorded as follows for negative reactions: 0, all RBCs at the bottom of the tube. For positive reactions: 1+, RBCs' agglutinates disperse in the gel but most at the bottom of the tube; 2+, RBCs' agglutinates disperse in the gel; 3+, some RBCs' agglutinates disperse in upper part and most are forming a red line on the top of gel; and 4+, all RBCs agglutinates form a red line on the gel's surface. Recipient plasma samples from Day 48 to Day 1657 crossmatched with Donor RBCs using AIC and AGC tests: strong to moderate reactions with AIC and 4+ incompatible reactions with AGC at Day 48 and 106. Weak reaction with AIC and 3+ incompatible reactions with AGC at Day 891 and 1657

### 3.2.5 | Anti-DEA 1 alloantibodies specificity in adsorption study

The presence of anti-DEA 1 alloantibodies in Recipient's plasma was assessed by crossmatching DEA 1+ Control RBCs against adsorbed plasma with the AIC technique. When previously adsorbed with DEA 1 – RBCs, the plasma from the DEA 1– Recipient showed the same variation of incompatibility against DEA 1+ Control RBCs (strip 3, Figure 6) as when not adsorbed (strip 2, Figure 6). In contrast, when previously adsorbed against DEA 1+ RBCs, Recipient's plasma became compatible with same DEA 1+ Control RBCs (strip 4, Figure 6).

## 4 | DISCUSSION

Among the known canine blood group systems, DEA 1 is considered clinically most important. However, little is known about structure or antigenicity of weakly to strongly DEA 1+ erythrocytes and onset and duration of sensitization with DEA 1+ blood.[17] In this transfusion study, we document strong and durable alloimmunization of a DEA 1 – recipient dog with blood from a weakly DEA 1+ and otherwise blood type matched donor dog after 16 days lasting for at least 4.5 years. Furthermore, alloantibodies against yet to be determined erythrocyte antigens were also induced, which appeared less strong and lasted less long. This study supports careful matching of DEA 1– recipients with DEA 1– blood and crossmatching of previously transfused dogs, whereas extended typing for other blood types for which blood typing reagents and/or tests are available will not be helpful.

Since the early experimental studies in the 1960s, it was recognized that DEA 1.1 was antigenic resulting in anti-DEA 1 alloantibody induction and acute hemolytic transfusion reaction when dogs were again transfused.[17,21] Furthermore, at least 1 clinical report documented sensitization of a DEA 1.1– dog with DEA 1.1+ blood.[11] The introduction of monoclonal anti-DEA 1 antibodies enabled the redefining of the DEA 1 blood group system with DEA 1– and weakly to strongly DEA 1 + blood types.[12] The degree of DEA 1 positivity was shown previously to remain stable overtime in a particular dog[13] and as also seen here with the weakly DEA 1+ Donor. In our previously published crossmatch survey, an accident mismatch of a DEA 1– recipient patient receiving a transfusion with strongly DEA 1+ blood became sensitized.[5] Here, we document that even weakly DEA 1+ blood transfused to a DEA 1– recipient induces anti-DEA 1 alloantibodies. Thus, weak DEA 1 typing

**TABLE 2** Major crossmatch (XM) results between Recipient's plasma and Control red blood cells (RBCs) after dog erythrocyte antigen (DEA) 1 mismatched transfusion according to DEA 1 type

| | Recipient plasma at Day 48 | | | Recipient plasma at Day 106 | | | Recipient plasma at Day 891 (2.4 years) | | | Recipient plasma at Day 1657 (4.5 years) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | XM | Compatible | Incompatible | XM | Compatible | Incompatible | XM | Compatible | Incompatible | XM | Compatible | Incompatible |
| Controls | n | n (%) | | n | n (%) | | n | n (%) | | n | n (%) | |
| DEA 1+ | 7 | 0 (0) | 7 (100) | 50 | 0 (0) | 50 (100) | 50 | 0 (0) | 50 (100) | 6 | 0 (0) | 6 (100) |
| DEA 1– | 4 | 0 (0) | 4 (100) | 38 | 14 (37) | 24 (63) | 38 | 29 (76) | 9 (24) | 5 | 2 (40) | 3 (60) |

*Notes*: Major crossmatch tests were made either with fresh or frozen Recipient's plasma samples depending of Controls RBCs availability at sampled dates. Availabilities of Recipient's plasma limited the number of crossmatches performed.

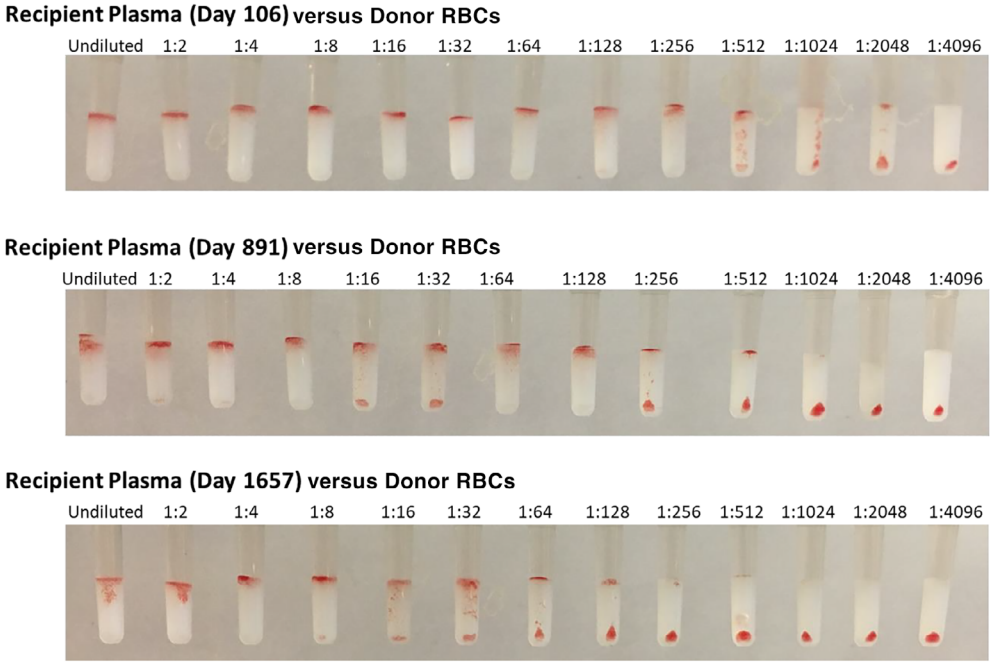


**FIGURE 4** Alloantibody titration between Recipient plasma and Donor red blood cells (RBCs) using the antiglobulin-enhanced gel crossmatch (AGC) technique. Recipient plasma samples from Day 106 to 1657 in serial 2-fold dilutions crossmatched with Donor RBCs. Agglutination reactions with AGC technique were recorded similarly as described in Figure 3

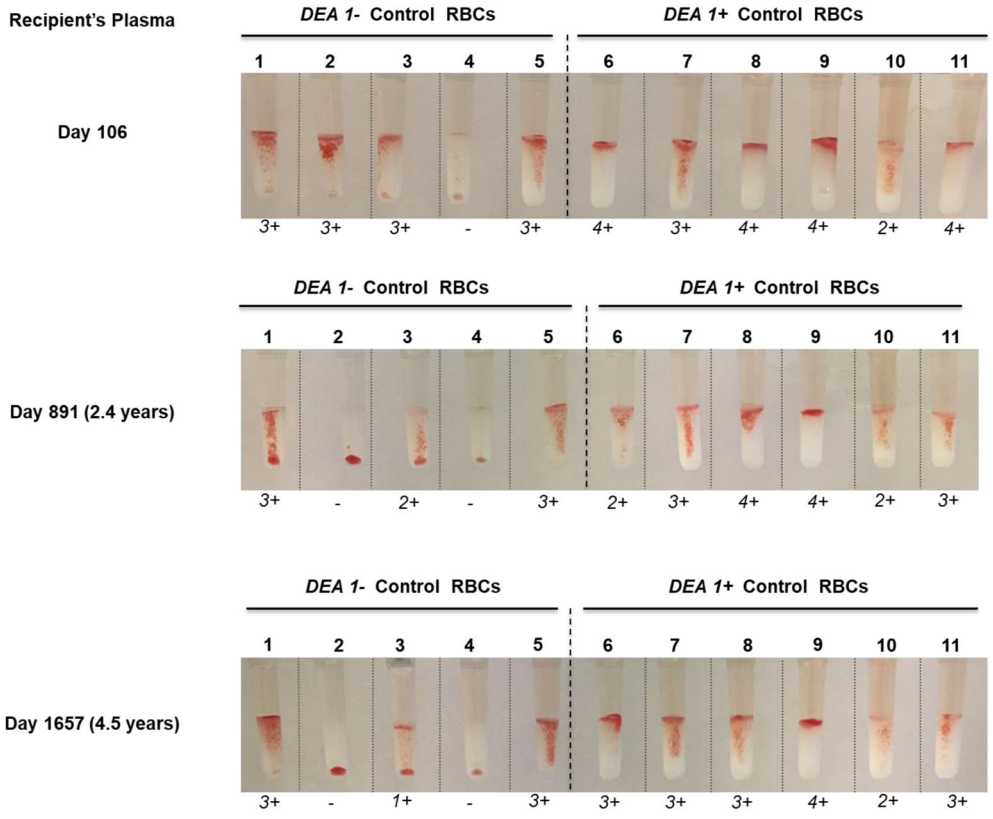


**FIGURE 5** Major crossmatch incompatibilities between Recipient's plasma and a panel of Control red blood cells (RBCs) using the antiglobulin-enhanced gel crossmatch (AGC) technique. Agglutination reactions with AGC technique were recorded similarly as described in Figure 3. Recipient's plasmas from Day 106 to Day 1657 crossmatched with Control RBCs using AGC displays 2+ to 4+ reactions against DEA 1+ Controls and negative to 3+ reactions with DEA 1– Controls

reactions by immunochromatography or card agglutination test must be recognized, and any weakly DEA 1+ donors must be labeled DEA 1+. It appears critical to type any recipient and donor and to not transfuse any DEA 1+ blood, as weak as the DEA 1 antigen could be, to a DEA 1– dog.

Although some naturally occurring anti-DEA 7 alloantibodies have been described in some studies,[7] others have reported consistently negative/compatible crossmatches of any donor with any recipient dogs which had not been previously transfused.[5] Furthermore, anti-DEA 7 alloantibodies do not appear to be of any clinical importance as not a single acute hemolytic transfusion reaction has been associated with DEA 7. In this study, neither the Recipient's nor the Donor's plasma contained any naturally occurring anti-DEA 7 alloantibodies (both were DEA 7–). Moreover, each time RBCs were available,

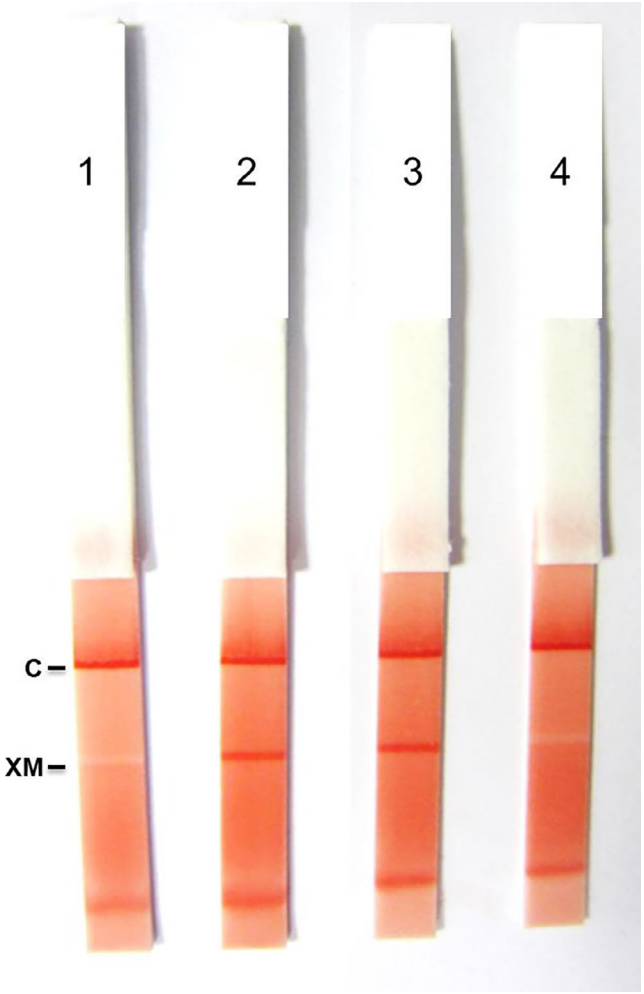


**FIGURE 6** Recipient's alloantibodies specificity study utilizing adsorbed recipient's plasma from Day 106 in antiglobulin-enhanced immunochromatographic strip crossmatch (AIC) tests. The presence of anti-dog erythrocyte antigen (DEA) 1 alloantibodies was assessed by crossmatching (XM) differently treated Recipient's plasma from Day 106 against the same DEA 1+ Control red blood cells (RBCs). Strip 1: Recipient autologous crossmatch (negative autocontrol). Strip 2: Crossmatch of DEA 1+ Control RBCs versus Recipient's plasma (positive control). Strip 3: Crossmatch of DEA 1+ Control RBCs versus Recipient's plasma previously adsorbed against DEA 1– Control RBCs. Strip 4: Crossmatch of DEA 1+ Control RBCs versus Recipient's plasma previously adsorbed against DEA 1+ Control RBCs. Antiglobulin-enhanced immunochromatographic crossmatch tests results were recorded similarly as described in Figure 3

1 tested DEA 1–, DEA 7+ Control was found to be compatible with Recipient's plasma from Day 106 to Day 1657. It should be noted that the DEA 7 antigen is not constitutive, but is adhering to canine erythrocytes.[17,22] Finally, none of the Control plasma samples revealed any incompatibilities in the minor crossmatch test confirming the lack of any alloantibodies before transfusion.

It is well recognized that after a first transfusion with canine blood, dogs can become sensitized, crossmatch incompatible as well as develop acute hemolytic transfusion reactions if again transfused.[5,9,11,23,24] The use of the same compatible donor in multiple transfusion cases will not assure compatibility even when originally testing compatible. Although it is generally recommended to start crossmatching dogs 4 days after a first transfusion, immunologically it is difficult to understand such fast alloantibody development and no studies have been done to systematically follow dogs after a transfusion in clinical settings. In this study, sensitization and alloantibodies were only detected >16 days but before receiving a third transfusion from the same Donor at Day 37. From an immunological perspective, this course of alloimmunization seems reasonable and concurs with studies in humans,[25] but does not negate the possibility of earlier alloimmunization in other recipients and against strongly DEA 1+ or other erythrocyte antigens in dogs.

Fewer data related to the canine erythrocyte antigen specificity causing a positive/incompatible crossmatch and or acute hemolytic transfusion reactions have been reported. Only reactions against DEA 1 (1.1), DEA 4, and Dal have been clinically documented.[11,16,23,24] Because the Recipient and the Donor were a match for all blood types tested except DEA 1 and were both DEA 3–, DEA 4+, DEA 5–, Dal+, and Kai 1+/2–, no reactions against these antigens should have occurred. However, the Recipient was initially sensitized against any donor tested independent of being DEA 1+ or DEA 1–. At later time points beyond 1 year, the recipient became compatible to some DEA 1– but never to DEA 1+ dogs. There is strong support for a DEA 1 sensitization with complete suppression of incompatibility with DEA 1+ dogs when Recipient's plasma is first adsorbed against DEA 1+ RBCs. Moreover, additional alloantibodies against undetermined and possibly yet to be determined erythrocyte antigens had to be assumed to explain the incompatibility reactions against DEA 1– cells that were found in this study by extensive crossmatching with many dogs. Hence, alloimmunization must have occurred against DEA 1+ and other erythrocyte antigens. Similarly, in a prior study and other unpublished observations by us and others, incompatible crossmatch results could not be associated with known erythrocyte antigens (blood types).[3,5] Furthermore, in most acute hemolytic transfusion reactions in dogs, the RBC antigen specificity remains elusive because of the limited availability of typing reagents and lack of characterization of canine blood group systems.

The duration of sensitization, that is persistence of induced alloantibodies, has not been studied in dogs. However, some crossmatch incompatibilities have been reported after weeks and at least 1 DEA 1 related acute hemolytic transfusion reaction occurred in a DEA 1– dog when again transfused with DEA 1+ blood 3 years after receiving untyped blood.[11] In this study, we documented persistent alloimmunization for 4.5 years against DEA 1 and also other undetermined erythrocyte antigens. The anti-DEA 1 alloantibody titer was and remained strong from Day 48 to the end of the study (Day 1657), whereas the undefined alloantibodies detected against DEA 1– RBCs appear to decline or even disappear. Alloantibody titration of the Recipient's plasma throughout the follow-up period correlated nicely with the decreasing intensity of incompatible crossmatch reactions. This further supports the strong antigenicity of DEA 1 even from weakly DEA 1+ dogs. The Recipient of this study reported here was transfused 3 times over a 37-day period, and hence we cannot assess if these alloantibodies would have formed

if the Recipient was only transfused once. We did not differentiate titers between IgG and IgM, but it would have been interesting to further characterize which class of induced Igs arose and disappeared in the Recipient's plasma. Finally, although it is anticipated that a DEA 1-related acute hemolytic transfusion reaction would occur, we do not know if the additional alloantibodies would be in vivo active and cause an acute hemolytic transfusion reaction.

## 5 | CONCLUSION

Dog erythrocyte antigen 1 typing before any transfusion and selection of DEA 1 matched or DEA 1– donors is recommended to prevent alloimmunization.[21] Weakly DEA 1+ blood is strongly immunogenic and thus weakly DEA 1+ donors should be classified as DEA 1+ dogs in any donor program.[12] The high rate of alloimmunization after transfusion outside the DEA 1 blood group system observed here and previously warrants major crossmatch testing in any dog that previously received RBC products >4 days before the next transfusion.[23,24]

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### CONFLICT OF INTEREST DECLARATION

M. Guidetti was employed, and I. Goy-Thollot and U. Giger have been scientific advisors to Dianov. Reagents, commercial typing and crossmatch kits were provided for these studies by Alvedia. The design and execution of the study, data analysis, and writing of the manuscript have been done independently. U. Giger is the director of PennGen at the University of Pennsylvania which is a non-for-profit laboratory offering blood typing and compatibility testing.

### OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

### INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Institutional Animal Care and Use and Ethics Committee of VetAgro Sup (#1267), and owner consent was obtained before Recipient and blood donor enrollment into the study and blood collection.

### HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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