FULL ARTICLE

ISSN Online 1678-4456

Blood typing in positive DEA 1 dogs: comparative analysis between immunochromatography, hemagglutination and flow cytometry

Tipificação sanguínea em cães AEC 1 positivo: análise comparativa entre a imunocromatografia, hemoaglutinação e citometria de fluxo

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ABSTRACT

Blood typing techniques have been improved to ensure greater safety for transfusion procedures. Typification for the DEA 1 antigen through flow cytometry should offer more reliability to routine immunohematology in donor and recipient dogs. Currently, the DEA 1 group is starting to be an autosomal dominant allelic system with the DEA 1 negative type and its variations of positivity. The present study investigated the DEA 1 antigen using the techniques of immunochromatography, hemagglutination and flow cytometry. Among the positive animals for the DEA 1 group, typified by flow cytometry, medium intensities of fluorescence were found, which are indicative of weak, moderate and strong antigenicity. This enabled the division of the DEA 1 group into weak positive, moderate positive and strong positive. The blood typing techniques for the DEA 1 group by flow cytometry, agglutination and immunochromatography had positive (Spearman r=0.70) and statistically significant (p>0.0001) correlations.

Keywords: Typification in dogs. Canine transfusion medicine. Transfusion risk in dogs. Dog erythrocyte antigen 1. Flow cytometry.

RESUMO

As técnicas de tipificação sanguínea vêm sendo aperfeiçoadas para garantir maior segurança aos procedimentos transfusionais. A tipificação para o antígeno AEC 1 com o emprego da citometria de fluxo poderá oferecer mais confiabilidade à rotina da imunohematologia em cães doadores e receptores. Na atualidade, o grupo AEC 1 passou a ser denominado como um sistema alélico autossômico dominante com o tipo AEC 1 negativo e suas variações de positividade. O presente trabalho comparou os resultados de três técnicas utilizadas para a pesquisa do antígeno AEC 1: cromatografia; hemoaglutinação e citometria de fluxo. Dentro dos indivíduos positivos para o grupo AEC 1, tipificados pela citometria de fluxo, foram encontradas intensidades médias de fluorescência indicadoras de antigenicidade fraca, moderada e forte, podendo-se dividir o grupo AEC 1 em positivo fraco, positivo moderado e positivo forte. As técnicas de tipificação sanguínea para o grupo AEC 1 por cromatografia, hemoaglutinação e citometria de fluxo apresentaram correlação positiva (Spearman r=0,70) e estatisticamente significativa (p<0,0001).

Palavras-chave: Tipagem em cães. Medicina transfusional canina. Risco transfusional em cães. AEC 1. Citometria de fluxo.

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Received: October 30, 2018
Approved: January 30, 2019

How to cite: Santos SCS, Santos MM, Rodrigues WF, Meyer R, Costa MFD. Blood typing in positive DEA 1 dogs: comparative analysis between immunochromatography, hemagglutination and flow cytometry. Braz J Vet Res Anim Sci. 2020;57(1):e151444. https://doi.org/10.11606/issn.1678-4456.bjvras.2020.151444

Introduction

In current veterinary medicine, the safe practice of blood transfusions is achieving more prominence and pre-transfusion screening standards have been established for donor animals. It is known that for a safe blood transfusion it is important to make use of typified and compatible blood (Giger, 2014; Tocci & Ewing, 2009).

According to Giger (2014), the DEA 1 system is an exception among other positive and negative systems, such as DEA 3, DEA 4, DEA 5, DEA 7 and Dal, then it is subdivided into DEA 1.1 (A1) and DEA 1.2 (A2), which are apparently allelic, and another allele that would be DEA 1.3 (A3). However, recent studies have indicated that it is more appropriate for DEA group 1 to be characterized as negative, weak positive, moderate positive and strong positive, rather than being subdivided into two or three subtypes (Euler et al., 2016; Lee et al., 2017).

Through recent typing by anti-DEA 1 monoclonal antibodies, the DEA 1 group is starting to be known as an autosomal dominant allelic system with the DEA 1 negative type and its variations of positivity (weak, medium and intense) (Acierno et al., 2014; Euler et al., 2016; Lee et al., 2017; Polak et al., 2015).

Evaluating the DEA 1 blood group when typing 53 positive DEA 1 dogs, Polak et al. (2015) subdivided it into positive weak DEA 1, positive moderate DEA 1 and positive strong DEA 1. Thus, it is consistent information that this model is dependent on an autosomal dominance with 4 to 5 alleles: AEC 1 negative (0), AEC 1 positive weak (1+), AEC 1 positive moderate (2+) and AEC 1 positive (3+ and 4+), comparing the findings of the reaction intensities of immunochromatographic strips to the mean values of fluorescence by the technique of typification by flow cytometry, a quantitative technique. Euler et al. (2016) also found a strong correlation between the visual and densitometric semi-quantitative results of the degrees of positivity of the AEC group 1 in weak, moderate and strong.

The blood typing of dogs, in general, is based on the serological identification by agglutination reactions. Originally, the serum of sensitized dogs was used for typing, through polyvalent alloantibodies, which vary among groups of animals due to the intensity of individual antigenicity of these animals (Chang et al., 2006; Giger et al., 2005). So, the recognition of the DEA 1 blood group is still made through polyclonal antibodies obtained by canine alloimmunization (Blais et al., 2007; Chang et al., 2006).

The present investigation analyzed the DEA 1 antigen using the flow cytometry and agglutination technique in 69 dogs of different breeds, aged between one and eight years, weighing from 28 kg up, and of both sexes, all positive by immunochromatography. Through the correlation between the three techniques, it is possible to identify each positivity score in DEA 1 positive animals, and to classify it as weak, moderate and strong. The blood typing techniques have been improved to ensure greater safety for transfusion procedures. The three techniques were compared to establish scores of the intensities of the reactions found in flow cytometry and agglutination in dogs positive by immunochromatography.

In veterinary transfusion medicine, the gold-standard technique for blood typing has not yet been established and the aim is to contribute to this goal.

Materials and Methods

In order to carry out the hemagglutination tests and the flow cytometry, samples of canine antisera from post-transfused dogs with DEA 1 blood type were submitted to the investigation of antibodies by flow cytometry as a follow-up of alloimmunization of these respective dogs (Santos et al., 2018). Each antiserum had its own average fluorescence intensity, ranging from 15 to 538.99 and representing the concentrations of anti-DEA 1 antibodies. When they were unified to form an antiserum pool, it presented a higher average fluorescence intensity, which was 979.52, demonstrating antiserum potentiation.

This antiserum pool was diluted in the ratio of 1:2, which was the titration that showed good hemagglutination reaction with blood known as DEA 1, because at higher dilution no hemagglutination was found in the crossmatching

tests in tubes. When submitted to the new evaluation by flow cytometry, it presented an average fluorescence of 623.24. This procedure was adopted so that no prozone effect occurred, with an antibody concentration higher than that of the antigen (Acierno et al., 2014; Hohenhaus, 2004; Stieger et al., 2005; Vap, 2010).

In order to perform the flow cytometry, the cytometer Becton Dickinson FACSCalibur™ interconnected to a PC Power Macintosh (Apple, Salvador, BA, Brazil, Laboratory of Immunology of the Federal University of Bahia) was used along with the instrument-specific software (BD CellQuest Pro™ software, Becton, Dickinson and Company).

The immunochromatography tests were performed using the commercial kit for the detection of DEA 1 blood group Alvedia® (Limonest, France), following the method provided by the manufacturer. Three drops of the buffer solution were placed in the tube, followed by 3 µL of whole blood with 5% EDTA. After homogenization for 7 sec, the samples were deposited in the strips for evidence of reaction or non-reaction followed by reading.

The samples tested underwent three washes with saline solution and a red cell concentrate of 4%. For one drop of the erythrocyte concentrate, two drops of the anti-DEA 1 polyclonal antiserum were added. This method followed the principle of reverse typing, in which the method of the test of crossmatching in tubes is carried out when the standard serum is an antiserum known as a specific anti-erythrocyte antigen. In this case, it contained anti-DEA 1 antibodies (Lacerda, 2005). The results were determined by the cross-intensity score, according to Gibson (2007).

For the flow cytometric typing of each blood sample, 2 mL of whole blood in EDTA was centrifuged at 2200 x g for 3 min. The plasma was discarded, and the red blood cell concentrate was resuspended with saline in the same volume as the discarded plasma. Subsequently, 2 µL of concentrate were mixed with 998 µL of saline solution and homogenized in vortex and then reserved for the test. 50 µL of the antiserum, interacting with the erythrocyte with the positive DEA 1 on its surface, and 50 µL of serum free from alloantibodies, representing the negative control, which did not interact with binding to erythrocytes DEA 1 positive or negative, were incubated at 56 °C for 10 min. Then there was a second incubation with 40 µL of the solution of erythrocytes to be tested diluted at room temperature for 30 min. The polyclonal anti-DEA 1 serum samples and the negative control with the erythrocyte suspension were washed with saline solution at 2200 x g for 3 min, 3 times. Positive and negative controls were also added to the vials, plus 3 µL of anti-dog IgG antibody at room temperature sheltered from light for 30 min. The sera were then twice washed with saline solution at 2200 x g for 3 minutes. Then, all vials were resuspended in 300 µL of saline and, finally, the readings were taken on the cytometer (Santos et al., 2018).

For the comparative analysis of the three techniques, a Spearman test was performed, and all variables were analyzed by Pearson's chi-square test. For the tables mean, standard deviation, median, minimum value, maximum value and variance by the program EXCEL, version 2013 were applied. The graphs were created using the GRAPH PAD PRISM program (GraphPad, 2018).

Results

During the standardization of positive and negative results, typing tests were performed by flow cytometry in animals that had already been typified by immunochromatography, where the lowest fluorescence mean value was 5.92 and the highest was 31.53 in nine negative animals that were being tested. For positive control, the flow cytometry technique was also performed in two animals known to be positive by immunochromatography, finding the mean fluorescence value of 979.52 and 623.24. Thus, the one with the highest value was chosen as positive control. Figure 1 shows the histograms of the negative and positive controls, while Figure 2 shows the histograms of the minimum and maximum fluorescence average results of the negative animals in the study, as well as the positive animals.

The group of 69 animals in the study were typified using the immunochromatography technique. Typing by flow cytometry and agglutination was also done, following the method of the crossmatching test in tubes, with the anti-seizure agent known as anti-DEA1. Of the 69 animals in this analysis typified by immunochromatography, 30 were negative and 39 were positive for the DEA 1 blood group. Table 1 shows the mean fluorescence and hemagglutination averages of the DEA 1 negative animals, where the fluorescence averages ranged from 5.92 to 71.46 and all presented negative hemoagglutination (Score 0). Table 2 shows the profile of the fluorescence averages of the DEA 1 positive animals, whose values ranged from 77.65 to 2391.82, and their agglutination scores were 1 to 3 crosses (1+, 2+, 3+) without any score 4 crosses (4+). The results of fluorescence averages in immunochromatography and flow cytometry of the 39 positive DEA 1 animals were compared with the agglutination scores and are presented in Table 3.

After establishing the respective cutoff points for positive and negative values, positivity and negativity were

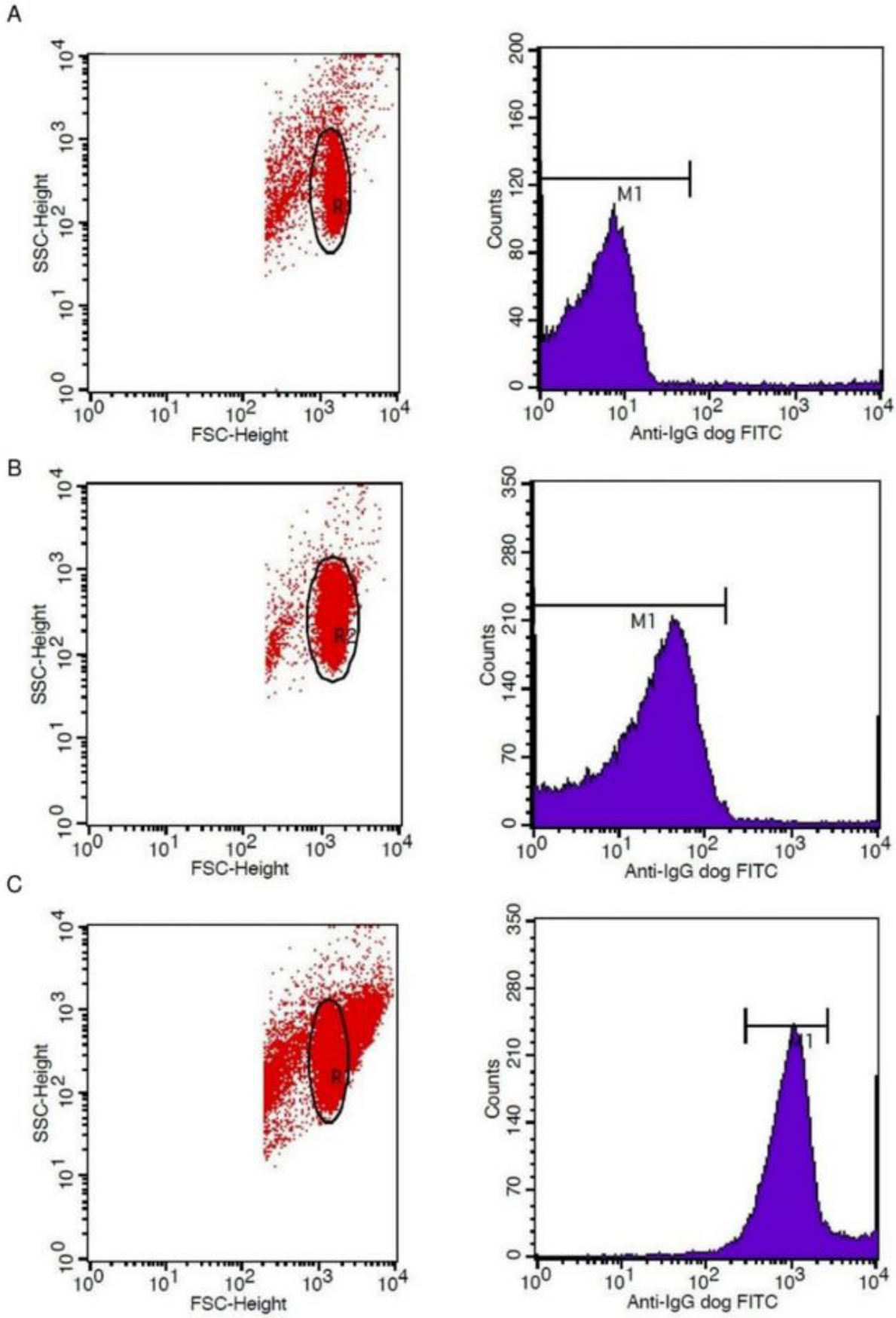

Figure 1 – Sequence of the negative and positive controls analysis with the primary anti-DEA 1 negative and positive sera in graphs of size and granularity of erythrocytes and individual histograms. (A) Negative control obtained with lower fluorescence mean (5.92) from negative erythrocytes for the DEA1 group; (B) Negative control obtained with the highest fluorescence mean (31.53) from negative erythrocytes for the DEA1 group, with DEA1 negative animal serum; (C) Positive control obtained from animal known from the DEA1 group (979.52), representing positive erythrocytes with DEA1 negative animal serum. M1 = Marker peak; FSC = Forware SCatter; FITC = Fluorescein Isothiocyanate.

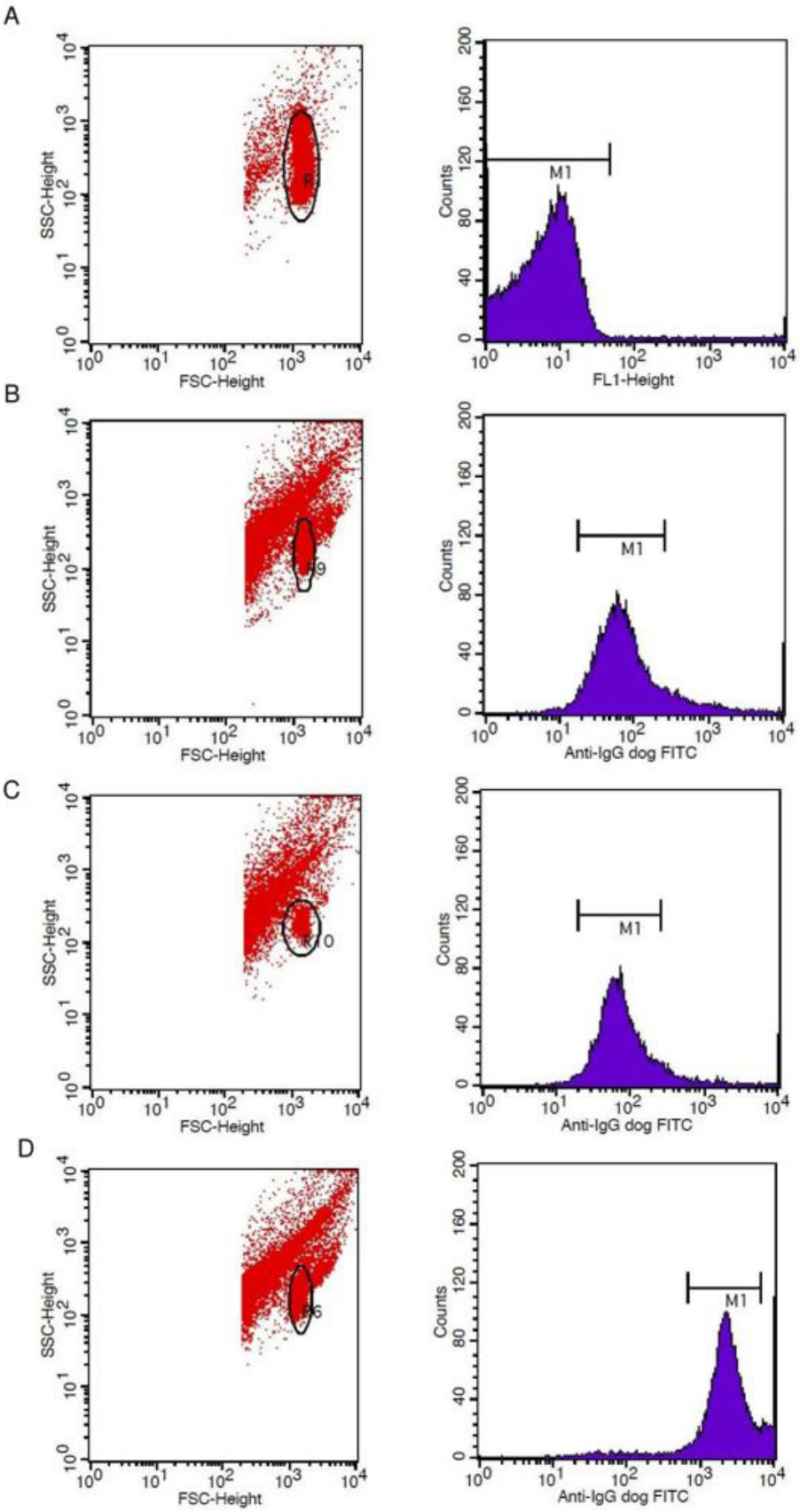

Figure 2 – Sequence of graphs in size and granularity of erythrocytes and individual histograms, of erythrocytes negative for animals studied and positive for group DEA 1, which presented minimum and maximum values of fluorescence. (A) Negative animal for the DEA1 group, showing the lowest fluorescence mean of the negative animal's group (7.67); (B) Negative animal for the DEA1 group, showing the highest fluorescence mean of the negative animal's group (71.46); (C) DEA1-positive animal with serum exhibiting the lowest fluorescence mean of the group of positive animals (77.65) and representing the fluorescence closest to the established cut-off point (71.47); (D) Positive animal for the DEA1 group, showing the highest fluorescence mean of the positive animal group (2391.82) M1 = Marker peak; FSC = Forware SCatter; FITC = Fluorescein Isothiocyanate.

Table 1 – Comparison of mean fluorescence intensity (MFI) values obtained in flow cytometry with hemagglutination (agglutination score 0, 1+, 2+, 3+, 4+) of the animals that had negative results in blood typing by the group immunochromatography DEA 1 (n=30) – Salvador – 2017

Animal	MFI	Agglutination
1	31.53	0
2	12.77	0
3	5.92	0
4	8.24	0
5	9.44	0
6	9.76	0
7	7.67	0
8	8.37	0
9	8.35	0
10	28.51	0
11	71.46	0
12	43.26	0
13	20.49	0
14	14.11	0
15	13.45	0
16	9.41	0
17	9.87	0
18	17.62	0
19	36.49	0
20	15.25	0
21	52.8	0
22	43.1	0
23	61.8	0
24	68.32	0
25	30.21	0
26	49.47	0
27	23.35	0
28	16.13	0
29	30.39	0
30	26.24	0
Average		26.126
Stardard Deviation		19.23265
Medium		11.43
Minimum		5.92
Maximum		71.46
Variance		369.8949

(n) = 30 animals. Average and Stardard Deviation (Pearson; p <0.0001).

Table 2 – Comparison of mean fluorescence intensity (MFI) values obtained in flow cytometry with hemagglutination (agglutination score 0, 1+, 2+, 3+, 4+) of the animals that had positive results in blood typing by the group immunochromatography DEA 1 (n=39) – Salvador – 2017

Animal	MFI	Aglutinação
1	119.48	1
2	140.4	1
3	266.27	3
4	1421.43	1
5	250.54	1
6	240.21	1
7	91.51	1
8	174.01	1
9	316.11	2
10	127.54	1
11	226.52	3
12	293.4	3
13	270.91	3
14	123.96	1
15	187.83	1
16	2391.82	3
17	688.98	2
18	348.25	2
19	377.76	2
20	688.21	3
21	704.6	2
22	1592.5	3
23	1627.38	3
24	593.86	2
25	865.08	2
26	94.99	1
27	222.5	1
28	85.93	1
29	142.48	1
30	145.11	2
31	113.98	1
32	293.4	2
33	270.91	2
34	161.75	1
35	81.35	1
36	174.24	2
37	467.84	2
38	213.73	2
39	77.65	1
Average		427.5492
Standard Deviation		509.4232
Medium		240.21
Minimum		77.65
Maximum		2391.82
Variance		259511.9

(n) = 39 animals. Average and Standard Deviation (Pearson; p <0.0001).

detected using the three different methods: flow cytometry, immunochromatography and hemagglutination, and they were compared for sensitivity (Figure 3). After evaluation of the data, no differences were found regarding the sensitivity of detection of positive and negative sera (p> 0.05).

Due to the variability of the agglutination intensity, as well as the values found in the immunochromatography, there was a correlation for the positivity between the different methods (Figure 4). After the comparisons, there was a positive (Spearman r = 0.70), and statistically significant (p <0.0001) correlation, regarding the sensitivity of the tests.

Table 3 – Distribution of animals with positive results in blood typing by DEA group 1, grouped by the obtained agglutination scores (1+, 2+, 3+), and then compared to the results of medium intensity of fluorescence (MIF) of flow cytometry (n=39) – Salvador – 2017

No. of DEA 1+ animals	Agglutination	MIF				Median	Variance
		Minimum	Maximum	Average	S. D.		
18	1+	77.65	1421.43	214.3078	305.9991	133.97	93635.48
13	2+	145.11	865.08	419.99	227.0188	348.25	51537.51
8	3+	226.52	2391.82	919.6263	836.2198	490.805	699263.6

(n) = 39 animals. Average and Standard Deviation (Pearson; p <0.0001); No = Number; S.D. = Standard Deviation.

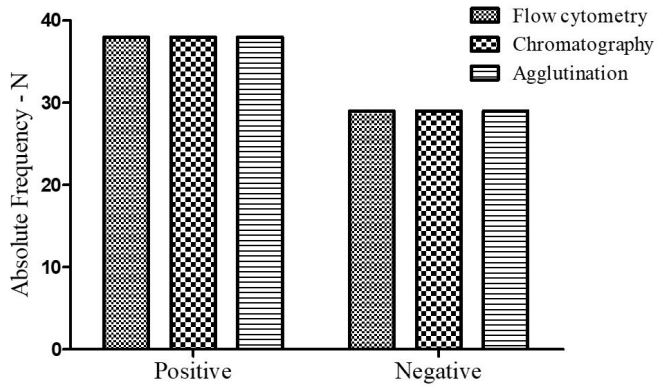

Figure 3 – Comparative analysis between the positive results of each technique for sensitivity. Note: After evaluation of the data, no differences were found regarding the sensitivity of detection of positive and negative sera (p> 0.05).

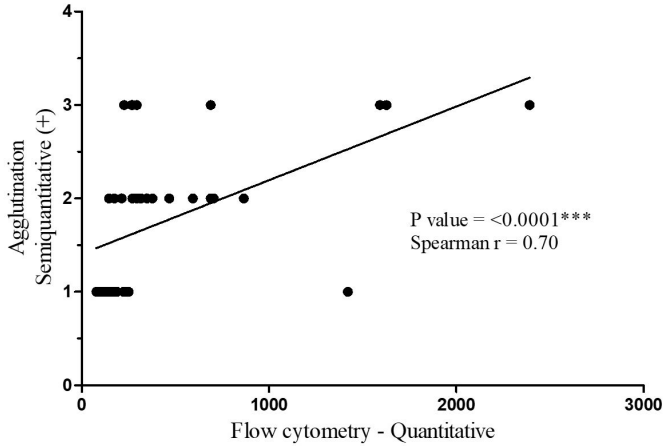

Figure 4 – Comparative analysis of quantitative values of positive hemoagglutination and flow cytometry. Note: After the comparisons, there is a positive (Spearman r = 0.70) and statistically significant (p <0.0001) correlation, regarding the sensitivity of the tests (***=p<0.0001).

Discussion

In the present work, the flow cytometry technique was used to detect the blood type of DEA 1 dogs. The results were compared with the immunochromatography technique and the hemagglutination technique.

Our results from the comparative analysis between immunochromatography, hemagglutination and flow cytometry were like those found by Acierno et al. (2014) when 66 dogs were typed by immunochromatography and flow cytometry, and the DEA 1 results in the immunochromatography were also positive for DEA 1 in flow cytometry. The Acierno et al. (2014) investigation used anti-DEA 1 murine monoclonal antibody as the primary antibody, conjugating to the polyclonal anti-murine secondary antibody of caprine origin, while in this study polyclonal antibody from alloimmunized dogs after blood transfusion with blood from the AEC 1 positive group was used as primary anti-DEA 1 antibody; and anti-canine polyclonal antibody of ovine origin was used as secondary antibody. Polyclonal primary antibodies were used demonstrating that the flow cytometry technique has high specificity as much as when using monoclonal antibodies.

The antiserum used here as a primary antibody in flow cytometry, which presented high concentration, was used as a polyclonal antibody to bind to the DEA 1 antigens in the crossmatching test in tubes (hemagglutination), and thus allowed to generate antigen-antibody reaction without the use of the Coombs canine antiglobulin reagent to obtain a better visualization of the agglutination. Blais et al. (2007) and Giger (2014) reported that initially the serum of sensitized dogs was used for typing, knowing that it was polyclonal serum, so that there could be variation between individuals and require the Coombs reagent for better visualization of the agglutination, but not always generating an excellent evaluation. In the present study, we did not observe in any sample the score of four crosses in agglutination, only the variation of one to three crosses, because in this method, the agglutinins produced have a poor reaction since we did not use Coombs reagent. Hara et al. (1991) identified the specificity of monoclonal antibodies when studying antibodies against type DEA 3, confirmed by cross-agglutination and using positive and negative erythrocytes. However, they found very low titles of 1:4 to 1:8 and with the reaction disappearing very quickly. In the present study, the maximum possible titration of the polyclonal antiserum for clumping was 1:2 without the aid of the Coombs reagent, like what Hara et al. (1991) found.

The immunochromatography tests showed binding results ranging from strong to weak, which is expected in this technique. However, it was preferable not to categorize reactions as weak, moderate and strong as this was a test where the intensity positivity is visual, which leads to subjectivity of interpretation. Unlike the immunochromatography method used by Acierno et al. (2014), in which it was possible to quantify an intensity score of 0 to 4 crosses (0+, 1+, 2+, 3+, 4+) by reading the test strips in a densitometer, where 0+ and 1 + were negative and the following positives increased by antigenic intensity.

When the flow cytometric analyses were performed, a variation in bond reactions was found, which is revealed by the fluorescence averages. When compared to the results obtained in the hemagglutination, which followed the method of the test of crossmatching reaction, it was possible to categorize groups of 1+, 2+ and 3+ scores that were found, correlating them as shown in Table 3. Acierno et al. (2014) argued, from the results obtained in their studies with the flow cytometry technique for the diagnosis of DEA 1 blood group, that it is more appropriate to use a typing scheme in positive DEA 1 and negative DEA 1, with the detection of its weak, moderate, and strong antigen expression, thereby eliminating the poorly defined patterning of DEA 1.2 and DEA 1.3 blood types.

Lucidi et al. (2011), typing blood samples by flow cytometry with anti-DEA 1.1 monoclonal antibody in 62 DEA 1.1 negative dogs by tube typing technique, found (MIF) of fewer than 25 (mean = 16 and median = 15) and 110 DEA 1.1 positive dogs also by tube typing found fluorescence averages greater than 73 (mean = 407 and median = 391). Goy-Thollot et al. (2017), typing DEA 1 animals with monoclonal antiserum, found an antigenic expression that they designated as negative DEA 1 which had less than 10 (MIF <10) in positive fraction of 10 ≤ MIF <100, moderate positive of 100 ≤ MIF <300 and strong positive with MIF ≥ 300. In the present study, the 30 negative animals tested using immunochromatography and agglutination in the flow cytometry had anti-AEC 1 polyclonal antibody lower than 71.46 (mean = 26.126 and median = 19.005); and the 39 positive animals, also using the same two techniques, had medium intensities of fluorescence higher than 77.65 (mean = 427.5492 and median = 240.21) in flow cytometry. When comparing the blood samples typed by immunochromatography with the same typified by flow cytometry, using the anti-DEA 1 primary antibody obtained by post-transfusion sensitization, a direct positive correlation was found as all presented positive results. Similarly, Euler et al. (2016), typing blood samples from dogs with monoclonal antibody by gel column, flow cytometry and immunochromatography, also obtained positive direct correlation results. This leads us to the conclusion that this antiserum had a high concentration of antibodies specific for the DEA group 1, even when it was an antiserum containing polyclonal antibodies. Comparing, Polak et al. (2015) carried out the re-establishment of animals considered to be negative DEA 1.1, positive DEA 1.1 and positive DEA 1.2 using monoclonal and polyclonal antibodies in parallel to determine their correlation. They found in positive DEA 1.1 animals a strong reaction when using antiserum to type DEA 1. In the same study, the negative DEA 1.1 samples tested by the monoclonal anti-DEA 1 antibody continued to present negative results, but when tested with polyclonal antibodies, weak to moderate DEA 1.1 were found. Surprisingly, six samples previously classified as positive DEA 1.2 were not in agreement with the results, with reactions of moderate variations with both the monoclonal and polyclonal reagents. In these samples, the results of moderate to strong positive DEA and negative DEA 1 were found.

Like the results from Lee et al. (2017) and Polak et al. (2015), the results found by flow cytometry in this work presented three groups of mean fluorescence intensity when compared with the scores obtained in agglutination. Lee et al. (2017), in a comparative study with the blood types Kai 1 and Kai 2, argued that the DEA 1 blood group is composed of a variation of DEA 1 types and not divided into DEA 1.1 and DEA 1.2. Polak et al. (2015), found it unusual to have two different proteins for DEA 1.1 and DEA 1.2 types as parts of the same blood group system. Simultaneously, through their flow cytometry research, they demonstrated that DEA 1.1 and DEA 1.2 are the same antigen and are not correlated with variations in group expression. It is a positive DEA 1 antigen with variations of weak, moderate and strong.

Safra et al. (2014), in preliminary studies of the canine genome, found only one nucleotide polymorphism for DEA 1 in the region of the CFA27 chromosome. Based on the findings of the immunoblotting tests and reactions with various monoclonal and polyclonal antibodies, Lee et al. (2017) suggested that the blood types Kai 1 and Kai 2 correspond to the proteins originally described as DEA 1.1 and DEA 1.2 and that the current monoclonal antibody DEA 1 recognizes a single erythrocyte antigen. These studies demonstrated that when polyclonal antibodies are used in the same way as in the present investigation, it is possible to find these three intensities of positivity as the authors described above as much as with the monoclonal antibodies, since it is a single blood group that is an autosomal dominant allelic system with the DEA 1 negative type and its variations of positivity.

The typification for DEA 1 antigen through flow cytometry should offer more reliability to routine immunohematology in donor and recipient dogs. This method has therefore been proposed as a gold standard within blood typing, ensuring more accuracy and reliability of such procedures.

Conclusions

The flow cytometry using polyclonal antibody for the blood typing of the DEA 1 group is an accurate technique with clear differentiation between the positive and negative results.

Among the positive individuals for the AEC group 1, typified by flow cytometry, there are medium intensities of fluorescence which are indicative of weak, moderate and strong antigenicity which can help to divide the AEC group 1 into weak positive, moderate positive and strong positive.

The blood typing techniques for the AEC group 1 by flow cytometry, agglutination and immunochromatography had a positive correlation (Spearman r = 0.70) and a statistically significant correlation ($p < 0.0001$).

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Ethics Statement

The present study was conducted in accordance with Law No. 11,794, on October 8, 2008, Decree No. 6,899, on July 15, 2009, and the norms issued by the National Council for the Control of Animal Experimentation (CONCEA), and was approved by the Ethics Committee on the Use of Animals ot the Institute of Health Sciences (CEUA-ICS), in a meeting on August 12, 2016.

Acknowledgements

This work was supported by the Diagnose Animal Veterinary Clinic in Bahia, the Immunology Laboratory of the Federal University of Bahia, and the Neurochemical Laboratory of the Federal University of Bahia, in Brazil.

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Financial Support: None.