The crawford variant as a cause of rhd typing discrepancies in blood banks: a case report

Sussan Barrera Margarita Bolívar Ayda Rodríguez Adriana Urbina

PII: S1246-7820(22)00037-4

DOI: https://doi.org/doi:10.1016/j.tracli.2022.03.006

Reference: TRACLI 3295

To appear in: *Transfusion clinique et biologique*

Accepted Date: 22 March 2022

Please cite this article as: Barrera S, Bolívar M, Rodríguez A, Urbina A, The crawford variant as a cause of rhd typing discrepancies in blood banks: a case report, *Transfusion clinique et biologique* (2022), doi: https://doi.org/10.1016/j.tracli.2022.03.006

THE CRAWFORD VARIANT AS A CAUSE OF RhD TYPING DISCREPANCIES IN BLOOD BANKS: A CASE REPORT

Sussan Barrera[a], Margarita Bolívar[a], Ayda Rodríguez[a], Adriana Urbina[b]

[a] Banco Nacional de Sangre, Cruz Roja Colombiana, Av Carrera 68 #68B-31, Bogotá, Colombia.

[b] Escuela de Medicina y Ciencias de la Salud, Ciencias Biomédicas, Universidad del Rosario, Carrera 24 #63C-69 Quinta Mutis, Bogotá, Colombia.

Sussan Barrera: sussan.barrera@cruzrojacolombiana.org

Margarita Bolívar: margaritabolivar28@hotmail.com

Ayda Rodríguez: ayda.rodriguez@cruzrojacolombiana.org

Adriana Urbina: Escuela de Medicina y Ciencias de la Salud, Ciencias Biomédicas, Universidad del Rosario, Carrera 24 #63C-69 Quinta Mutis, Bogotá, Colombia. Email: adriana.urbina@urosario.edu.co. Phone: +57-2970200, extension 3319.

THE CRAWFORD VARIANT AS A CAUSE OF RhD TYPING DISCREPANCIES IN BLOOD BANKS: A CASE REPORT

LA VARIANTE CRAWFORD COMME CAUSE DES DIVERGENCES DE TYPAGE RHD DANS LES BANQUES DE SANG: UN RAPPORT DE CAS

ABSTRACT

We present the case of a 55-year-old Colombian male who showed a discrepancy in the serological typing of the RhD antigen in his first platelet donation. The discrepancy persisted after a serological investigation with multiple Anti-D monoclonal reagents (IgG and IgM) under different conditions (22°C and 37°C, saline, and LISS/Coombs). Furthermore, partial RhD typing was performed, obtaining negative results with a commercially available panel of six Anti-D reagents. Molecular analysis showed a homozygous deletion of *RHD* and heterozygosity for the Crawford variant *(RHCE*ce, RHCE*ceCF),* with a predicted phenotype of C-, c+, E-, e+, Vs+, V+. Following the investigation of this case, this man has made 14 platelet donations showing variable reactivity, with agglutinations ranging from - to 2+. Since Crawford red blood cells express some RhD antigen epitopes, they could cause alloimmunization in RhD negative receptors. Likewise, Anti-D alloantibodies have been documented in Crawford variant carriers. Therefore, it is recommended that carriers of this variant be classified as RhD positive if they are blood donors and RhD negative if they are transfusion recipients. Also, in pregnant women carrying a Crawford variant, Anti-D immunoprophylaxis is recommended.

RÉSUMÉ

Nous présentons le cas d'un homme de 55 ans, colombien, qui a montré une divergence dans le typage sérologique de l'antigène RhD lors de son premier don de plaquettes. La différence a persisté après une étude sérologique avec plusieurs réactifs monoclonaux Anti-D (IgG et IgM) dans diverses conditions (22°C et 37°C, Coombs saline et LISS). De plus, un typage RhD partiel a été réalisé avec un panel commercial de six réactifs Anti-D et des résultats négatifs ont été obtenus. L'analyse moléculaire a montré une délétion homozygote pour le gène *RHD* et une hétérozygotie pour la variante de Crawford (*RHCE*ce, RHCE*ceCF*), avec un phénotype prédit C-, c+, E-, e+, Vs+, V+. Pour donner suite à l'enquête sur ce cas, cet homme a fait quatorze dons de plaquettes, pour lesquels une réactivité variable a été observée avec des agglutinations de - à 2+ avec le même réactif Anti-D. Étant donné que les globules rouges de Crawford expriment certains épitopes de l'antigène RhD, ceux-ci pourraient provoquer une allo-immunisation contre les récepteurs RhD négatifs. De même, des alloanticorps Anti-D ont déjà été documentés chez des porteurs de variantes de Crawford. Par conséquent, il est recommandé que les porteurs de cette variante soient classés comme RhD positif s'ils sont donneurs de sang et RhD négatif s'ils sont récepteurs de transfusion. Finallement, pour les femmes enceintes porteuses d'une variante de Crawford, l'immunoprophylaxie Anti-D est recommandée.

KEYWORDS

Rh blood group system; Blood donors; Apheresis donation; Blood typing; Serologic typing; Crawford variant.

MOTS-CLÉS

Système de groupe sanguin Rh; Donneurs de sang; Don d'aphérèse; Typage sanguin; Typage sérologique; Variante de Crawford.

1. Introduction

The Rh system has been recognized as one of the most complex blood group systems due to the number of antigens (at least 56) [1], immunogenicity, and antibody heterogeneity. Although the introduction of two different Rh nomenclatures reflected different hypotheses about number of genes encoding these antigens, it is now accepted that the genes involved are *RHAG*, *RHD*, and *RHCE* [2]. The *RHD* and *RHCE* genes originated from gene duplication; therefore, they are very similar to each other, only differing in 30 to 35 amino acids [3]. In addition, these genes are highly diverse, due to deletions, gene conversions and nonsense mutations, leading to antigenic variants that may have clinical implications [3].

Some variants encoded by the *RHCE* gene can lead to the expression of some RhD antigen epitopes (EpD) reacting with certain monoclonal Anti-D reagents and adding greater complexity to D antigen typing. For example, the R_O^{Har} variant (D^{HAR}, RH33), found in some people of German descent, and the Crawford variant (ceCF, RH43), found in people of African descent and Colombian subjects, deserve special attention because they show strong reactivity (3+ to 4+) with some monoclonal reagents while not agglutinating with others (including an indirect antiglobulin phase testing) leading to discrepancies in RhD typing [4]. In the R_O^{Har} (RH33) carriers, the exon 5 of *RHCE* is substituted by the correspondent exon of *RHD*, leading to the expression of some D antigen epitopes (Ep5 and Ep6/7), which explains why their erythrocytes react with certain monoclonal Anti-D reagents [5,6]. In the Crawford variant (ceCF, RH43), the Q233E and L245V mutations in exon 5 of the *RHCE* gene lead to the expression of some D epitopes, and a W16C mutation in exon 1 lead to the expression of the Vs (RH20) antigen [7,8]. Other *RHCE* variants, such as *ceRT (RHce R154T)* and *ceSL (RHce S122L)*, are also characterized by mutations leading

to the expression of antigens that "mimic" some D epitopes, resulting in weak reactivity with certain monoclonal Anti-D reagents [8].

We present the case of a first-time platelet donor in whom RhD typing showed a discrepancy using various Anti-D reagents and in whom genotyping determined that he was a carrier of the Crawford variant. The donor gave informed consent, and the institutional ethics committee endorsed the publication of this case report.

2. Case report

A 55-year-old apparently healthy man, a native and resident of Bogotá (Colombia), made a first-time platelet donation in July 2019. At the time of donation, the blood bank was conducting a comparison study of two automated immunohematology analyzers, and the sample was processed in two analyzers with different reagents for the ABO and RhD typing, and for irregular antibody screening. A discrepancy was found in the RhD typing, with a negative result (-) in one analyzer and a weak positive result (+/-, 2+, 3+) in the other. Therefore, additional blood samples were obtained from the donor, and supplementary serological and molecular analyses were performed.

2.1. Initial blood typing

Mandatory blood bank immunohematological tests were performed simultaneously using two different platforms with the same EDTA-whole blood samples. On the one hand, in an IH-1000 (Bio-Rad) analyzer, with six-column cards, the following reagents were used: DiaClon ABO/D+Reverse Grouping for Patients (A: A5; B: G1/2; D: LHM59/20 (LDM3) and 175-2); C3d (C139-9) with ID-DiaClon Anti-D (ESD1) reagents in a LISS/Coombs

card, for pooled irregular antibody screening and weak D antigen determination at 37 ° C; DiaClon Rh-Subgroups + K (C: MS-24, c: MS-33, E: MS-260, and MS-49, MS-21, MS-63, K: MS-56); and ID-DiaCell I-II-III and ID-DiaPanel. On the other hand, in an Erytra analyzer (Grifols), with eight-column cards, the following reagents were used: DG Gel® ABO/Rh (2D) (A: 16243 G2 and 16247 E6; B: 9621 A8; AB: 16245 F11 D8, 16247 E6, 7821 D9; D: P3x61; D´(VI+): P3x290, P3x35, P3x61, P3x21223 B10); Serascan Diana 2/3; DG Gel Coombs (12011 D10); DG Gel Rh Pheno + Kell (DVI^+: RUM-1 and ESD-1M; C: MS-24; E: DEM-1; c: H-48; e: MS-21, MS-63 and MS-16; Cw: MS-110; K: MS-56); and Identisera Diana.

The direct and reverse ABO typing consistently showed that the donor was group A (Figures 1 and 2). However, a discrepancy was observed in the RhD typing results. The result was RhD negative in the IH-1000 analyzer (Bio-Rad) with an Anti-DVI^- (Figure 1A). In contrast, the result was D positive with a weak agglutination in the Erytra analyzer (Grifols): 2+ with Anti-DVI^-, and +/- with Anti-DVI^+(Figure 2). Therefore, the Rh and Kell phenotype was determined, and the result in both platforms was C-, c +, E-, e +, Kell-. However, in the Erytra analyzer (Grifols), whose cards have eight columns, the result was negative with anti-Cw and RhD positive (3+) with an Anti-DVI^+ (Figure 2B). The irregular antibody screening was negative on both platforms.

2.2. **Serological investigation**

Given the persistent discrepancy in the RhD typing, a further serological investigation of partial RhD variants was carried out using a six monoclonal Anti-D panel, in LISS/Coombs at 37°C (ID-Partial RhD Typing Set, reference 001451, Bio-Rad), with

clones LMH76/55 (IgG), LMH77/64 (IgG), LMH70/45 (IgG), LMH59/19 (IgG), LMH169/80 (IgG) and LDM1 (IgM), obtaining negative results (Figure 3). A summary of the donor's red blood cell reactivity with different monoclonal Anti-D reagents is presented in Table 1.

2.3. Molecular analysis

Additional blood samples were obtained, which were sent to the State University of Campinas (Unicamp, Campinas, SP, Brazil), for DNA microarray analysis of 35 alleles (exons 1-7 and 9) associated to *RHD* variants (*wRHD* BeadChip®, Bioarray Solutions, Immucor), showing negative results. Additionally, blood samples were sent to the Grifols Immunohematology Center (Grifols IH Center, San Marcos, TX, United States) for *RHD* and *RHCE* gene sequencing, showing homozygous deletion of *RHD (RHD**deletion, *RHD**deletion) and heterozygosity for the Crawford variant (*RHCE*ce, RHCE*ceCF*), with three mutations in the *RHCE* gene: 48G/C (W16C), 697C/G (Q233E) and 733C/G (L245V), and a predicted phenotype of C-, c+, E-, e +, Vs+, V+.

2.4. Variable behavior in subsequent donations

After his first platelet donation, the donor made 14 donations between 2019 and 2020. Variable reactivity was found in RhD typing with the IH-1000 (Bio-Rad) platform using anti- DVI^- with LHM59/20 (LDM3) and 175-2, showing agglutinations from - to 2+, while in the indirect antiglobulin phase of testing (Anti-IgG and C3d) with an Anti-D ESD1 were always negative (Table I).

However, results of weak D antigen determination at 37°C, and C3d () with Anti-D () have remained negative (Table 1).

3. Discussion

We report the case of a 55-year-old Colombian man who showed a discrepancy in the serological typing of the RhD antigen using different Anti-D reagents in his first platelet donation. After a serological investigation using multiple monoclonal Anti-D reagents, both IgG and IgM, under different conditions (22°C and 37°C, in saline and LISS/Coombs), the discrepancy persisted. Nor could a partial D variant be identified using a commercially available panel of monoclonal reagents. Instead, molecular analysis showed homozygous deletion of *RHD* and heterozygosity for the Crawford variant (*RHCE*ce, RHCE*ceCF*), with a predicted phenotype of C-, c+, E-, e+, Vs+, V+. After investigating this case, this man has donated platelets 14 more times and has shown variable reactivity (from - to 2+) using the same Anti-D reagent. Since the strength of expression of some erythrocyte antigens can decrease with sample storage, the time elapsed until serological testing was considered. It was found that the storage times of the samples varied from less than one hour to 44 hours, but there was no relationship with the intensity of agglutination with Anti-D.

The Crawford variant (ceCF, RH43) is characterized by three mutations in the *RHCE* gene that lead to the expression of some D epitopes (EpD). These mutations are 48G/C (W16C), 697C/G (Q233E), and 733C/G (L245V), which lead to the expression of V and Vs antigens, and D epitopes 6.1, 6.2, and 16.1 [8]. It is precisely the expression of these D epitopes that leads to positive reactions with some monoclonal Anti-D reagents, especially

GAMA401 (IgG), RUM1 (IgM), F5S (IgG), H2D5H2D2 (IgG), and MCAD6 (IgG) [8,10]. Because of this, some of the reagents used, which contain RUM1, showed reactivity. However, according to the diverse Anti-D reagents presented in Table I, reactivity was also observed with other reagents not previously reported to identify the Crawford [10–12].

The Crawford variant was initially described by Cobb in 1980 [11] and later by Esteban et al. in a pregnant Colombian woman [13]. However, its serological and molecular characterization was only carried out years later from a case series including African Americans and Colombians [7,8]. Moreover, in the United States, a Crawford variant frequency of 0.7% in RhD negative people of African descent, or 0.007% in the general population, has been estimated [8]. Although most of the available reports have been made from patients, a series of cases of four blood donors in the United States and new Anti-D reagents capable of recognizing this variant have also recently been reported [12]. In this case report, we describe other Anti-D reagents with this ability and show that the intensity of agglutination with the same Anti-D reagent is time-varying in the Crawford variant. Therefore, it would be important in future studies to evaluate the antigenic density in this variant and its behavior over time. Additionally, although several of the reports of the Crawford variant have been Colombians, we do not know its population frequency in the country, so it would be worth making this estimate.

It has been suggested that the Crawford variant, like other *RHCE* variants such as R_O^{Har}, ceRT, and ceSL, may have low immunogenicity due to the limited number of expressed D epitopes and a low number of variant RhCE proteins on the blood cell membrane.

However, the number of antigenic determinants per cell has not been evaluated for any of these variants. In contrast, at least two cases of patients with this variant have been reported in whom an Anti-D alloantibody was documented after the transfusion of RhD-positive red blood cells [8]. Although we conducted a retrospective follow-up on 16 patients transfused with the products from the blood donor with Crawford variant, all these recipients were RhD positive, so it was not possible to estimate the occurrence of RhD alloimmunization. Thus, while the immunogenicity of Crawford when transfused to RhD negative recipients is determined, it is recommended that the carriers be classified as RhD positive if they are blood donors and as RhD negative for transfusion purposes. Likewise, Anti-D immunoprophylaxis should be implemented for pregnant women [8,14]. Finally, to increase the sensitivity for detecting the RhD antigen and its variants in blood banks, different isotypes (IgM and IgG) and clone mixtures of Anti-D reagents, including those for DVI^{+} variants, should be used.

ACKNOWLEDGEMENTS- To the technical and administrative staff of the Colombian Red Cross National Blood Bank for their support to this case report. To Biocientifica LTDA. for its support in the serological research, including the panel for typing partial RhD variants, and in submitting samples to Unicamp (Campinas, SP, Brazil) for molecular analysis of the *RHD* gene. To Annar Health Technologies for providing the Erytra equipment and reagents (Grifols) for ABO and Rh blood typing and irregular antibody screening, and for sending the samples for *RHD* and *RHCE* gene sequencing (Grifols IH Center, San Marcos, TX, United States). To Professor Adrien Morel, from the Genetics and Genomics Research Group at the Universidad del Rosario (GENIUROS) for reviewing the abstract in French.

AUTHOR CONTRIBUTIONS- Sussan Barrera and Margarita Bolívar carried out the serological investigation and submitted the samples; Sussan Barrera and Adriana Urbina prepared the manuscript; Margarita Bolívar and Ayda Rodríguez reviewed the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST- The authors declare that they do not have any conflicts of interest.

REFERENCES

[1] ISBT. Table of blood group antigens within systems, v10.0 30-JUN-2021. Red Cell Immunogenetics and Blood Group Terminology | ISBT Working Party n.d. https://www.isbtweb.org/isbt-working-parties/rcibgt.html (accessed March 20, 2022).

[2] Shaz BH. Chapter 23 - Rh Blood Group System. In: Hillyer CD, Shaz BH, Zimring JC, Abshire TC, editors. Transfus. Med. Hemost., San Diego: Academic Press; 2009, p. 123–7. https://doi.org/10.1016/B978-0-12-374432-6.00023-3.

[3] Avent ND, Reid ME. The Rh blood group system: a review. Blood 2000;95:375–87. https://doi.org/10.1182/blood.V95.2.375.

[4] Westhoff CM. The Structure and Function of the Rh antigen Complex. Semin Hematol 2007;44:42–50. https://doi.org/10.1053/j.seminhematol.2006.09.010.

[5] Beckers EA, Porcelijn L, Ligthart P, Vermey H, Von dem Borne AE, Overbeeke MA, et al. The RoHar antigenic complex is associated with a limited number of D epitopes and alloanti-D production: a study of three unrelated persons and their families. Transfusion 1996;36:104–8. https://doi.org/10.1046/j.1537-2995.1996.36296181919.x.

[6] Beckers EA, Faas BH, von dem Borne AE, Overbeeke MA, van Rhenen DJ, van der Schoot CE. The R0Har RH:33 phenotype results from substitution of exon 5 of the RHCE gene by the corresponding exon of the RHD gene. Br J Haematol 1996;92:751–7. https://doi.org/10.1046/j.1365-2141.1996.382918.x.

[7] Schlanser G, Moulds, MK, Flegel, WA, Wagner, FF, Frame, T. Crawford (Rh43), a low incidence antigen, is associated with a novel RHCE variant RHce allele, ceCF. Transfusion 2003;43:34A.

[8] Flegel WA, Wagner FF, Chen Q, Schlanser G, Frame T, Westhoff CM, et al. The RHCE allele ceCF: the molecular basis of Crawford (RH43). Transfusion 2006;46:1334–42. https://doi.org/10.1111/j.1537-2995.2006.00901.x.

[9] Wagner FF, Ladewig B, Flegel WA. The RHCE allele ceRT: D epitope 6 expression does not require D-specific amino acids. Transfusion 2003;43:1248–54. https://doi.org/10.1046/j.1537-2995.2003.00495.x.

[10] Moulds, MK, Schlanser G, Frame, T. Reactivity of Monoclonal Anti-D Reagents With 27 Samples Shown to Have the Crawford(Rh43) Low-Incidence Antigen and/or a novel RHCE Variant Rhce Allele. Transfusion 2003;43:7A.

[11] Cobb,ML. Crawford: Investigation of a new low frequency red cell antigen. Transfusion 1980;20:631.

[12] Anani WQ, Gorlin J, Denomme GA. Anti-D selection for D assignment among pregnant women and blood donors: impact of the Crawford antigen. Transfusion 2020;60:1378–80. https://doi.org/10.1111/trf.15803.

[13] Esteban, R, Nogues, N, Montero, R. RhD epitope expression in a RHD negative individual: characterization of a novel RHCE variant allele. Transfus Clin Biol 2001;8:8S.

[14] Rikabi, N, Krugh, DW, Kennedy, M. Rh(D) Typing Discrepancy Similar to Ro HAR. Transfusion 2003;43 (Suppl):96A.

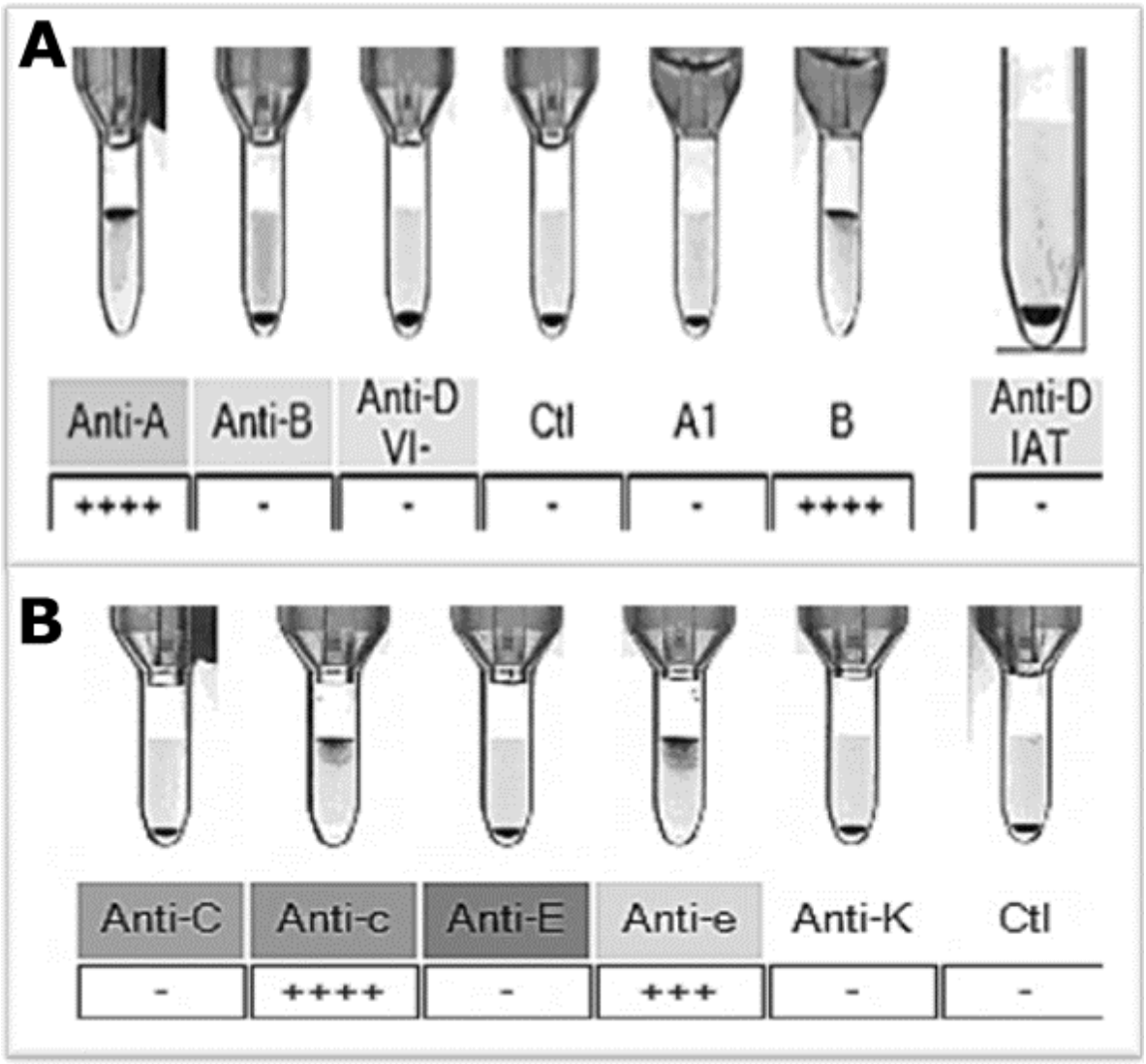

Figure 1. Serological typing at the initial donation, performed with IH-1000 (BioRad). **A)** ABO and RhD typing by immediate spin testing with an Anti-DVI^{-} LHM59/20 (LDM3) and 175-2, and indirect antiglobulin phase of testing (Anti-IgG and C3d) with an Anti-D ESD1, with the result RhD negative. **B)** Rh and Kell phenotype, with result C(-), c(4+), E(-), e(4+), K(-).

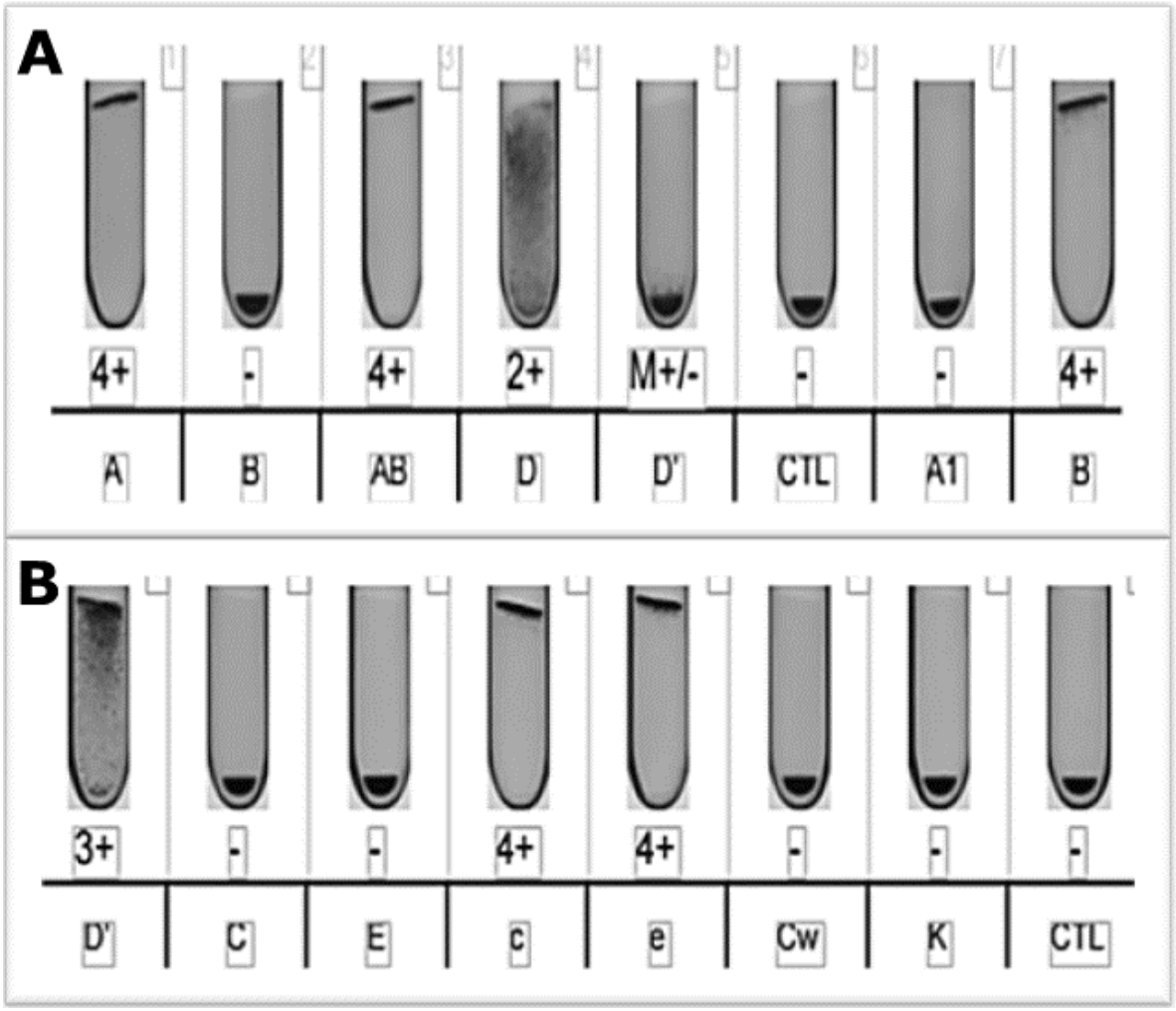

Figure 2. Serological typing at the initial donation, performed with Erytra (Grifols). **A)** ABO and RhD typing. In the fourth column the Anti-D was P3x61, with 2+ agglutination; in the fifth column the Anti-D´(VI^{+}) mixture included P3x290, P3x35, P3x61, and P3x21223 B10D, with +/- agglutination; the results were interpreted as RhD positive with weak agglutination. **B)** Rh and Kell phenotype, with result C(-), c(4+), E(-), e(4+), Cw(-), K(-), and D(3+); the Anti-D VI+ mixture included RUM-1 and ESD-1M.

LHM 76/55	LHM 77/64	LHM 70/45	LHM 59/19	LHM 169/80	LDM1
-	-	-	-	-	-

Figure 3. Partial RhD serological typing. A panel of six Anti-D reagents (ID-Partial RhD Typing Set, Bio-Rad) in LISS/Coombs phase at 37° C was used, with a negative result.

Table 1. Crawford variant (ceCF, RH43) red blood cell reactivity with different Anti-D reagents.

Monoclonal Anti-D	**Isotype**	**Temperature**	**Phase**	**Agglutination in gel column**
LHM 59/20 (LDM3) 175-2	IgM	22°C	Saline	Variable in 15 donations: 8/15 (53.3%) showed no agglutination (-); 4/15 (26.7%) showed agglutinations 1+; and 3/15 (20.0%) showed agglutinations 2+.
P3x61	IgM	22°C	Saline	2+
P3x290, P3x35, P3x61, P3x21223 B10	IgM/IgG	22°C	Saline	+/-
RUM-1, ESD-1M	IgM	22°C	Saline	3+
TH-28, MS-26, 175-2	IgM/IgG	22°C	Saline	2+
ESD-1M, 175-2	IgM/IgG	22°C	Saline	2+
TH28, RUM-1, LDM1	IgM/IgG	22°C	Saline	1+
LDM1	IgM	22°C	Saline	-
ESD1	IgG	37°C	LISS/Coombs	-
LMH76/55	IgG	37°C	LISS/Coombs	-
LMH77/64	IgG	37°C	LISS/Coombs	-
LMH70/45	IgG	37°C	LISS/Coombs	-
LMH59/19	IgG	37°C	LISS/Coombs	-
LMH169/80	IgG	37°C	LISS/Coombs	-
LDM1	IgM	37°C	LISS/Coombs	-